DNA REPAIR AND CELLULAR AGING IN MAMMALS

By

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Repair of DNA damage is a process that is expected to be crucial in setting the level of the error content of cellular components. Errors in cellular components are expected to be deleterious to normal cellular functions. Since maintenance of normal function is expected to be prerequisite for longevity, DNA repair is likely to be a determinant of the life span of an organism.

If the accumulation of errors in cellular macromolecules plays a role in mammalian senescence, this could also affect those components involved in DNA repair. An impairment of DNA repair systems with age would imply an increasing incidence of unrepaired or misrepaired damage to the genome with age. The purpose of this work was to determine, therefore, if DNA repair is altered with age in rats.

Skin fibroblasts derived from young (3 weeks old) and aged (2 years old) Fischer 344 rats were treated with either 1.70 mM ethylnitrosourea, 4 micromolar N-acetoxy-2-acetyl-aminofluorene, or 1.35 micromolar benzo(a)pyrene-anti-diol-epoxide. Following treatment, the kinetics of DNA excision repair and the elongation of newly synthesized, undamaged daughter DNA strands were followed using the alkaline elution technique for determination of single-strand DNA size, and the total, overall rate of DNA synthesis was determined.

Significant differences were found between cells of young and old rats in the formation and resealing of single-strand breaks in parental DNA and the elongation of undamaged, daughter strand DNA after both alkylation and arylalkylation damage. These results are consistent with decreased efficiency of DNA repair in cells derived from aged rats.

The relation of experimentally induced DNA damage to that expected to occur by natural mechanisms, *in vivo*, is discussed as are those aspects of DNA repair and the possible modulation of DNA repair expected to be important for future aging research.

CHAPTER 1
INTRODUCTION

It is apparent that the genetic makeup of an organism is a major determinant of the life span of that individual organism. That this is so is evidenced by the fact that the variation among the life spans of individuals of the same species is less than the variation among different species. It follows that in order to determine why some species live longer than others, we must know something about the differences in genetic make-up between species of differing longevity. Obviously we cannot examine every gene product of even one species. The problem, then, is to decide which functions are most likely to be critical determinants of longevity.

It seems reasonable to assume that specific functions are reponsible for maintenance of viability in all the cells of an organism. Based on the rate of evolution of the increase in life span for the human ancestral line, these functions may be few in number.[1] With age these functions might decline in efficiency, and this in turn would impair the proper functioning of the cells. If this were true, we might expect that, eventually, the failure of some critical organ system would lead to the demise of the organism. This, of course, is consistent with what we know of death in mammals. With this in mind we can see that the key to longevity might be processes necessary for the maintenance of proper function in _all_ cells.

Two questions can be asked. First, what are these processes, and, second, do they deteriorate with age?

Ideas about the events leading to the dysfunction and ultimate death of cells, and therefore of organisms, can be categorized as either error theories or programmed death theories.

Probably the best known proponent of the error accumulation hypothesis is Orgel.[2] He originally suggested that errors produced in components of the protein synthesizing system would cause that system to become more error-prone. Production of defective proteins such as RNA polymerases would increase the error content in newly made proteins. The positive feedback for error-proneness would cause an irreversible build-up of mistakes until the cell could no longer survive. In dividing cells this would also apply to the DNA synthesizing system. Error containing DNA polymerases would cause the synthesis of polymerases containing more errors until finally, the newly synthesized DNA would no longer be functional.

This could also be applied to mitochondria since they are known to synthesize some of their own components. However, a large number of errors in mitochondria would be contributed by the nuclear-cytoplasmic system. Even errors in those components coded for by the mitochondrial genome might not be independent of cytoplasmic influence since some of the components necessary for mitochondrial transcription and translation are probably made in the cytoplasm.[3]

Also, we must not underestimate the effect of errors in other cellular components.[4] If the proper functioning of the cell is dependent upon the integrity of all its components, then errors in any of these components would result in decreased efficiency of other components. For instance, defects in the cell membrane could be imagined to alter the availability of amino acids and, therefore, affect protein synthesis. This might result in the synthesis of incomplete, and therefore defective, proteins or failure to replace proteins that have become faulty. This same idea could be extended to extracellular components. The cells of the body are responsible for constructing and maintaining their own environment. If this environment becomes defective, then it would be expected to have an adverse effect on the cells.

Probably the major stimulus for proposing some sort of program or counting mechanism in normal cells that accounts for their finite life span has been the need to account for the fact that transformed cells in culture are capable of apparently infinite growth while normal, diploid cells are capable of only a limited number of population doublings.[5] If normal cells have a mechanism to count off the number of cell divisions, this could be interrupted or destroyed in transformed cells.

Holliday[6] has pointed out, however, that there are several lines of evidence which argue against this proposal. If untransformed cells had some kind of counting mechanism that determined the number of cell divisions before death, one might not expect environmental influences to alter their division potential. Nevertheless, exposure of fibroblasts to ionizing radiation,[7] elevated temperatures or 5-fluorouracil decreases their division potential.[6] Also, one might expect that at any given point in the life span of a culture of fibroblasts, all cells would be capable of the same number of mitoses. However, cells taken from normal cultures show heterogeneity in growth potential.[8]

Orgel[2] actually hinted at a mechanism involving the accumulation of errors and cellular selection by division that would account for the observed differences in growth potential of normal, diploid fibroblasts and transformed cells. This idea has been elaborated further by Holliday.[6] Consider that for any cell type there is a probability, P, that the accumulation of errors in the cellular components will reach a critical level such that the development of a higher error level is inevitable. This build-up of errors would be irreversible, and the cell would be committed to a sequence of events leading to death. In slowly dividing cells, the initial accumulation of the critical level of errors would not lead immediately to cell death because undamaged components would be present in sufficient quantities. At any given P, as a population of cells of this type aged, more and more cells would accumulate errors until, eventually, virtually all of the cells would contain an error level equal to or greater than the critical level. All cells in the population would then be irreversibly committed to death. In rapidly dividing cells, the initial build-up of errors to the critical level would be lethal because the supply of undamaged components in these cells would be less than that required for life. Those cells that reached the critical error level would die leaving only the uncommitted cells in the population. In a rapidly dividing population, there would be a continual selection for relatively undamaged cells. Growth of the population could thus continue indefinitely.

Three things would be involved in determining mortality or immortality for a population of cells. As P decreased, or as the mitotic rate increased, or as the size of the population increased, the growth potential of the population would increase. In the latter case, the more cells there were in the slowly dividing population, the longer it would take before all the cells became committed to death.

Since the tissues of an organism are composed of various cell populations, these considerations apply to cellular aging which occurs in vivo. On the basis of these arguments, then, mammalian senescence could be explained solely by the accumulation of errors in cellular constituents.

Orgel later concluded, however, that the error content of cellular components should eventually reach a plateau level rather than increase infinitely.[2] This is because an error containing system should be able to synthesize a new system containing fewer errors.[2] For instance, suppose an error containing mRNA is being translated by a faulty translation system. Since the translation system is making mistakes, there is a finite probability that a message error will be translated incorrectly to insert the correct amino acid into the newly forming peptide. This same concept could be applied to all of the statements made above about transcription and other cellular consituents. Experimental evidence for this hypothesis has been obtained with Escherichia coli. When E. coli were treated with streptomycin, the error level in flagellin proteins increased within a few generations to a stable, plateau level while essentially all the cells remained viable.[9] However, these were growing cultures, and the observed, overall, average, error level could well have been below the critical level for these cells. As long as the plateau level of errors is below the critical level for any given cell, that level of errors would be consistent with the immediate, continued viability of the cell. Orgel's revised hypothesis can thus be seen, by this mechanism, to fit the scheme proposed by Holliday.

Considerable evidence has been accumulated indicating that the level of error containing macromolecules does increase in the cells of older organisms.[10-45] Although in no case is it known whether the observed error level approaches the critical level for any given cell type, the fact that increased error levels are observed indicates that the error levels in the cells of older individuals are closer to the critical levels than in the cells of younger individuals. This is consistent with Holliday's proposal which predicts that these cell populations will eventually senesce.

The observed correlation of decreased replicative life span of fibroblast cultures with increased donor age[46] is also consistent with Holliday's proposal. These observations have been extended to include other parameters such as rate of fibroblast migration, cell population replication rate, cell number at confluency,[46] and colony size distribution.[47]

Day has suggested that tissue culture cells might be in a constant state of error prone (see Results and Discussion) DNA repair as a result of overwhelming stresses induced by "fluorescent light, nutrient imbalances, sublethal production of peroxides or accumulation of waste products between feedings".[48] We now know, for instance, that common fluorescent light induces mutations[49] and chromosome damage[50] in tissue culture cells. I would suggest further that altered parameters observed in cultures derived from aged donors reflect decreased capacity of in vivo aged cells to withstand a variety of stresses whether they be damage to DNA or to other cellular components. This could be due to a higher error level in aged donor cells resulting in decreased ability to mount an effective response to damaging insults.

Key processes which maintain and protect normal functioning of cells would be those that serve to keep the error level below the critical level. Among those expected to accomplish this are specific removal of damaged proteins,[51-53] scavenging of harmful radical species,[54] and repair of DNA damage.[55]

This study has focused on DNA repair since increased capacity for this function has clearly been shown to be correlated with increased life span. Hart and Setlow[56] found that the ability of fibroblasts to carry out U.V.-induced unscheduled DNA synthesis is correlated with the log of the life span for several mammalian species. This was confirmed by Sacher and Hart[57] using fibroblasts derived from the rodent species Mus musculus and Peromyscus leucopus. The latter lives 2.3 times longer than the former and has 2.3 times the repair capacity as evidenced by U.V.-induced unscheduled DNA synthesis. There was no difference, however, in the rate of rejoining of X-ray-induced single-strand breaks in DNA of fibroblasts from M. musculus and P. leucopus. It is tempting to speculate, therefore, that longevity might be correlated with increased capacity for repair of DNA base damage as opposed to repair of single-strand breaks.

It is of interest to know if DNA repair capacity decreases with age. If so, this would be reflected in an increased error level in the DNA with age. Decreased functional capacity for DNA repair in the face of an increased error level in DNA might indicate the development of a positive feedback for increased error production in this system analogous to that proposed by Orgel. Because of the central importance of the genetic information contained in DNA, accumulation of unrepaired or misrepaired DNA damage would have a pervasive, adverse effect on the functioning of each cell and, therefore, of the entire organism. For this reason, then, I have studied repair of DNA base damage as a function of age.

Evidence for accumulation of DNA damage with age in mammals includes increased levels of single-stranded regions[27,28,35] in DNA of mice, increased levels of DNA single-strand breaks in dog brain[29] and mouse liver,[43] decreased double-strand molecular weight of rat liver DNA,[30] increased incidence of chromosome abnormalities in rats,[31] mice,[40] dogs,[41] guinea pigs,[42] and humans,[32,38,39] apparent loss of rRNA genes in brain DNA of dogs,[34] and accumulation of DNA-protein crosslinks with age.[37,44] Curtis and Miller[42] showed that the incidence of spontaneous chromosome aberrations not only increases with age but that the rate of increase is inversely proportional to maximum life span when mice, guinea pigs, and dogs are compared. This correlates well with the studies by Hart and Setlow and Sacher and Hart mentioned above.

Certain facets of DNA repair are known not to change with age. Wheeler and Lett[29] found no change with age in the ability of _in vivo_-irradiated canine cerebellar neurons to repair X-ray-induced single-strand DNA breaks. This observation has been repeated in rabbit retina _in vivo_ and in primary fibroblasts derived from rabbits of different ages.[58] Ono and Okada[59] have obtained similar results _in vivo_ with mouse cerebellum, spleen, and liver. No changes were found in single-strand break rejoining ability in spite of the increased levels of single-strand breaks found in unirradiated dog cerebellum[29] and mouse liver.[59] Also, the aged cells _in vivo_ were apparently able to rejoin newly-formed, X-ray-induced, single-strand breaks but not those that had already accumulated with age. Accumulation of single-strand breaks with age may represent accumulation of end groups rendering those breaks inaccessible to the repair systems.

Yielding[60] has suggested that accumulation of DNA damage with age may result from inaccessibility of certain unused portions of the genome to repair enzymes. In dividing cells all of the genome would be used at one time or another since the entire genome must be replicated for a cell to divide. In nondividing, terminally differentiated cells such as muscle cells and neurons only those portions of the genome necessary for the function peculiar to that cell would be used. To maintain viability for these cells only these relatively few areas of the genome need be maintained error free.

Some evidence for preferential repair of different regions of the genome comes from studies indicating higher levels of repair replication in DNA that is more rapidly released by micrococcal nuclease digestion.[61-62] These studies did not determine if a higher level of damage was initially introduced into nuclease accessible regions, thus accounting for the greater repair seen there. This was ruled out, however, by Ramanathan[63] et al. and Wilkins and Hart.[64] Ramanathan used labeled carcinogens and observed preferential removal of this label from DNAase I accessible regions after long incubation times in rat liver. Wilkins used a micrococcal repair endonuclease extract to observe a greater percentage removal of U.V.-induced endonuclease sites from chromatin not shielded by protein than from those shielded by protein. Feldman et al.,[65] however, found similar extents of removal of labeled benzo(a)pyrene adducts from micrococcal nuclease accessible and inaccessible regions of DNA in chromatin.

Further evidence for areas of preferential repair comes from cytological studies. Berliner et al.[66] and Harris et al.[67] both found that the distribution of repair DNA synthesis after U.V. light and a variety of chemical carcinogens differs from the distribution of DNA in the nucleus. However, the two studies disagree on the location of the greatest repair DNA synthesis; it being found at the periphery of the nucleus in the former and in the central region in the latter study. These differences may be due to the different cell types used and/or to different methods used to determine DNA distribution within the nucleus.

Yielding's scheme also predicts that as cells differentiate to a nondividing state, their repair response to DNA damage should decrease. This is substantiated by considerable experimental evidence. Differentiation of myoblasts into mature myotubes is associated with decreased repair response after treatment with U.V. light,[68] methylmethanesulfonate,[69] or 4-nitroquinoline-1-oxide (4-NQO).[70] This is found in cultures of chick and rat myoblasts and the L6 myoblast cell line, respectively. It is interesting to note that rejoining of X-ray or 4-NQO-induced single-strand breaks was not decreased upon fusion of L6 myoblasts to form myotubes. On the other hand, the murine C-1300 neuroblastoma cell line does lose the capacity to rejoin X-ray-induced single-strand breaks[71] as well as the capacity to repair U.V.-induced damage upon differentiation.[72] Also, chick neural cells lose, upon differentiation, the capacity to repair methylmethanesulfonate and N-acetoxy-2-acetylaminofluorene-induced damage.[73]

These changes may simply reflect differences in repair capacity between dividing and nondividing cells. Lymphocytes stimulated to divide, for instance, are known to have an increased repair response to U.V. light[74] and N-acetoxy-2-acetylaminofluorene-induced[75] damage over that found in unstimulated cells.

The repair response in terminally differentiated cells is not zero; just less than that in the undifferentiated, dividing state. It is tempting to speculate that differentiated, nondividing cells still repair those regions of the genome necessary for proper functioning but allow the rest to slip into disrepair. A process such as this may be an additional factor in the accumulation of DNA damage with age in nondividing cells. Additionally, it could provide a safety mechanism against cell division in tissues such as mature muscle and nervous tissues where cell division is strictly disallowed. Accumulation of nonrepairable breaks as observed in the above studies[29,59] might create a block to DNA replication and therefore prevent the cell from dividing.

A more fruitful area of investigation may be changes in repair of DNA base damage that occur with age. A start has been made in this direction. Schneider and Kram have examined sister chromatid exchange (SCE) formation after mitomycin C (MMC) damage using human fibroblasts in culture[76] and mouse and rat bone marrow cells in vivo.[77] After high doses of MMC, decreased levels of SCE's were observed with increased donor age. Sister chromatid exchanges are believed to be related in some way to DNA repair although the exact nature of this involvement is not at all clear.[78] Since MMC is a bifunctional alkylating agent, these studies may indicate decreased repair of DNA-DNA and/or DNA-RNA and/or DNA-protein crosslinks. Lambert et al.[79] have studied U.V.-induced DNA repair synthesis in human peripheral lymphocytes and found a decrease with donor age. This was not found, however, in a study of U.V.-induced repair synthesis in human spermatocytes as a function of age.[80] Lehmann et al.[81] have studied postreplication repair (see Results and Discussion) of U.V.-induced damage in fibroblasts derived from aged humans and found no alteration. Finally, Bochkov and Kuleshov[82] and Deknudt and Leonard[39] have studied chromosome aberrations after treatment of human lymphocytes with the alkylating agents N,N',N"-triethylene thiophosphoamide or dihydrochloride-1,6-di-(chlorethyl)-

amino-1,6-desoxymannitol and X-rays, respectively, and found an increasing incidence of induced aberrations with age. This might imply decreased DNA repair with increased donor age.

Until the significance of SCE's are more clearly understood it will be difficult to interpret Schneider and Kram's results conclusively. It is clear that more information is needed about repair of base damage and aging in terms of different types of base damage and in terms of different facets of the response to DNA damage. The experiments reported here were designed to obtain additional information of this type.

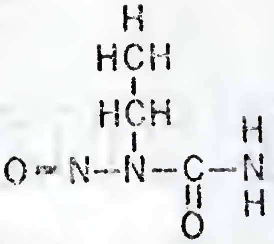
CHAPTER 2
EXPERIMENTAL DESIGN

For the work reported here a technology that would provide as much information as possible about DNA metabolism following DNA damage was desirable. Sensitivity was a prime consideration so that low levels of damaging agents could be used. Response to DNA damage could then be measured at low cytotoxicity. Since chemical agents would be used to damage DNA (see below), the use of unlabeled chemicals was desirable so as to avoid the high cost of labeled mutagens. The alkaline elution technique developed by Kohn and associates[83-84] was seen to provide all these advantages. As described in detail in Methods and Materials, this technique allows a sensitive measure of the degree of single-strand fragmentation of DNA. Fragmentation of DNA below 1-1.5x10^9 daltons can be detected. This is considerably higher than the upper limit of 5x10^8 daltons obtainable with alkaline sucrose gradient techniques.[83]

By labeling DNA of growing fibroblasts with ^{14}C thymidine before chemical treatment of the cells, fragmentation and resealing of long-term labeled, parent strand DNA, which occurs during prereplication excision repair, could be monitored. By giving a pulse of ^{3}H thymidine to these same cells at various times after chemical treatment, elongation of undamaged, newly synthesized DNA strands could be followed. In these experiments this latter synthesis occurred opposite a previously damaged, parental template. Finally, by comparing the total amount of pulse ^{3}H thymidine to the total ^{14}C thymidine taken up by the cells during the long term pre-labeling period, a measure of the overall rate of DNA synthesis at various times after chemical treatment could be obtained.

Since this was to be a study of DNA repair as a function of _in vivo_ aging, appropriate animals were needed as a source of skin fibroblasts. A long-lived strain with no abnormal pathology was required in order to assure that the results reflected _natural_ aging processes.[85] Female, Fischer 344 rats were chosen largely because of the availability of aged animals through the National Institute on Aging. The youngest and oldest animals available, three weeks old and two years old, were used in order to maximize the chances of detecting differences that might exist.

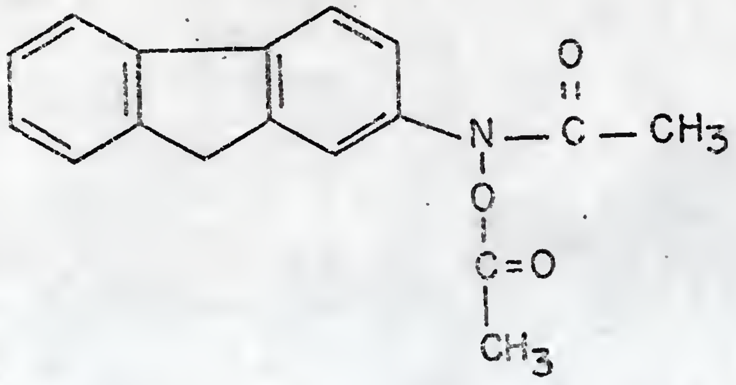
The damaging agents used were N-ethyl-N-nitrosourea (ENU) (figure 1), N-acetoxy-2-acetylaminofluorene (N-AAAF) (figure 2), and benzo(a)pyrene-anti-diol-epoxide (BPADE) (figure 3). A variety of DNA adducts are produced by ENU (figure 1).[86] The best studied of these are N7-ethylguanine, N3-ethyladenine, O6-ethylguanine and ethyl phosphotriesters. The N3-ethyladenine and O6-ethylguanine adducts are enzymatically removed from DNA while N7-ethylguanine is lost largely by spontaneous depurination.[87] Ethyl phosphotriesters are not removed from DNA.[88-89] Two adducts are produced by N-AAAF (figure 2).[90] These are thought to be enzymatically removed from the DNA of human and rodent fibroblasts.[91] Figure 3 lists the adducts usually found in DNA after treatment with BPADE.[92]



N-Ethyl-N-Nitrosourea

Derivative	% of Total Ethylation
O6-EtGuanine	7
O2-EtThymine	6
O4-EtThymine	1.4
O2-EtCytosine	0.5
Ethyl Phosphotriester	70
N7-EtGuanine	(14)
N3-EtAdenine	(3)

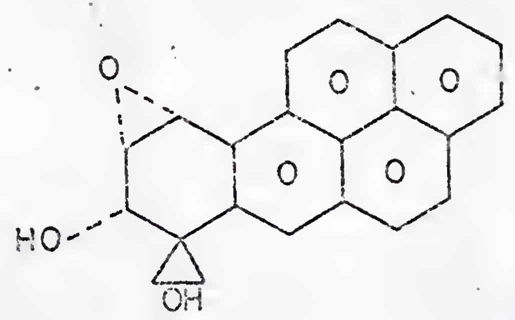
Figure 1. Structure of ENU and the percentage distribution of the adducts formed by ENU in double-stranded DNA. Parentheses indicate an approximate division of the 17% total N-ethylguanine adducts based on earlier work by the same author.



N-acetoxy-2-acetylaminofluorene

Derivative	% of Total
N-(deoxyguanosine-8-yl)-2-acetylaminofluorene	85
3-(deoxyguanosine-N^2-yl)-2-acetylaminofluorene	15

Figure 2. Structure of N-AAAF and the distribution of the adducts formed by N-AAAF in DNA (see ref. 89).



7β, 8α -dihydroxy-9α , 10α -epoxy-7, 8,9-10-tetrahydrobenzo(a)pyrene

Derivatives

N^2-(7β,8α,9α -trihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene-10-yl)deoxyguanosine

N^6-(7β,8α,9α -trihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene-10-yl)deoxyadeosine

Figure 3. Structure of BPADE and the adducts usually found in DNA after treatment with BPADE.

The major guanine adducts are removed to some extent from rodent[93] and human[65] cells. In addition, a similar adduct is formed at the N7 position of guanine which is apparently very unstable and leads to rapid depurination from the DNA.[94]

Alkylation and arylalkylation adducts are expected to represent different classes of DNA damage since they would create different degrees of distortion of the DNA double helix.[55] For example, Heifich has shown that treatment with N-AAAF or BPADE creates S1 nuclease sensitive sites in DNA whereas N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) treatment does not.[95] (The adducts created by MNNG are similar to those from ENU. A methyl group is transferred by the former, an ethyl group by the latter. Distortion of the DNA helix is expected to be very similar for the two.) Less helix distortion may result from the respective N2-arylalkylguanine adducts since it is the N6-arylalkyladenine[96] adduct of BPADE and the C8-arylalkylguanine[90] adduct of N-AAAF that give rise to S1 nuclease digestible regions.

These results do not imply that ENU-induced damage has no effect on DNA architecture. Alterations of base pairing and secondary structure are expected to result from alkylation by ENU, and this has been demonstrated experimentally for O6-ethylguanine,[97] and for O4-ethyluracil and O2-ethyluracil adducts.[98] However, the arylalkyl adducts apparently produce much greater distortion.

Repair of these two different classes of damage might be analogous to repair of base damage due to ionizing radiation versus repair of U.V.-induced pyrimidine dimers. The existence of the genetic conditions Xeroderma Pigmentosum (exhibiting excision repair of ionizing radiation-induced base damage[99] but not of pyrimidine dimers)[100] and Ataxia Telangiectasia (exhibiting normal excision repair of pyrimidine dimers but defective excision repair of gamma radioproducts)[101] suggests that these two classes of DNA damage may require different repair systems.[55] Additionally, Xeroderma Pigmentosum cells may be able to repair MNNG-induced damage[102] but not N-AAAF-induced damage.[103] Ataxia Telangiectasia cells can repair N-AAAF-induced damage[103] but not MNNG-induced damage.[104] Therefore, the genetic definition of these two classes of DNA damage can be extended to include the alkyl and arylalkyl adduct damage employed in the studies reported here. Unfortunately, recent evidence indicates that this classification of DNA damage and repair is too simple. Pyrimidine dimers and N-AAAF damage may be removed from mammalian DNA by separate repair systems having different rate-limiting steps.[103,105]

As research continues it is inevitable that further distinctions will be made. It is possible that one or the other of these repair systems may contribute preferentially to senescence.

CHAPTER 3
METHODS AND MATERIALS

Animals

Three week old, female, pathogen free, Fischer 344 rats were obtained from Charles River Breeding Labs. Age upon arrival was checked by comparing the weights of the animals against a normogram provided by the breeder. Upon arrival the rats weighed 35-40 grams each. They were housed in the animal care facilities of the J. Hillis Miller Health Center for 1-2 days before obtaining tissues for culture.

Two year old, pathogen free, female, Fischer 344 rats were obtained from Charles River Breeding Labs through the National Institute on Aging. These were from a special colony of rats maintained for aging studies. The two year old rats were not checked for age upon arrival since no normogram was available for this age group. They were simply shipped from Charles River at two years of age. They arrived at our facility weighing approximately 270 grams each and were housed for 2-3 days before tissue samples were obtained. The mean 50% life span for female, Fischer 344 rats is very close to that for males which is reported to be 29 months.[106]

General Tissue Culture Methods

All cultures were maintained in Eagle's minimum essential medium containing 70 mg penicillin G and 50 mg streptomycin per liter plus 15 mM HEPES, pH 7.4, (MEM) plus additions as indicated. All cultures were kept in a humidified 37°C incubator with a 90% air plus 10% CO_2 atmosphere.

All trypsinization was done with a solution of 0.1%(w/v) trypsin in versene which was filtered through two 0.2 micron pore Nalgene filters before use.

All sera were heat inactivated at 56°C for 45 minutes before use.[107]

Phosphate buffered saline (PBS) is 0.15 M NaCl + 0.005 M K_2HPO_4, pH 7.4. Versene is 0.137 M NaCl + 1.15 mM KH_2PO_4 + 2.68 mM KCl + 8.10 mM Na_2HPO_4 + 0.478 mM Na_4EDTA, pH 7.4.

Isolation Of Tissue Samples

Tissue samples for the initiation of fibroblast cultures were obtained by the following method.

After washing with 70% ethanol, the ears of an anesthetized animal were clipped off with scissors and bathed for two minutes in 70% ethanol contained in a sterile petri dish. They were then rinsed for two minutes in sterile PBS also contained in a sterile petri dish. The ears were then split into two pieces and allowed to soak in the freezing medium (MEM + 10%(v/v) glycerol) for 10-20 minutes prior to freezing in a liquid nitrogen bath. All these procedures were carried out in a laminar flow, sterile, tissue culture hood.

For storage the samples were held in the vapor phase of a liquid nitrogen refrigerator.

Initiation Of Fibroblast Cultures

Rat ears were thawed, placed in a sterile petri dish containing 10-20 ml MEM + 0.1%(w/v) collagenase, and sliced with a sterile scalpel into pieces approximately 3-4 mm on a side. The ear pieces plus media were transferred to a sterile, square, glass bottle with a magnetic stirrer bar in the bottom. This was then placed in a 37°C warm room and allowed to stir for 45-60 minutes. (The square shape of the bottle seemed to aid separation of the tissues by physical agitation during digestion.) After digestion the contents of the bottle were filtered through sterile gauze into a screw cap, conical centrifuge tube and centrifuged for 10 minutes at 1000 rpm in a model PR-2, refrigerated International centrifuge. The cell pellet was resuspended in 10 ml MEM + 10%(v/v) fetal calf serum (FCS) and transferred to a T-25 (25 cm^2 growth area) plastic tissue culture flask. Due to the different sizes of three week old and two year old rat ears, one three week old ear gave rise to one such T-25 culture, and one two year old ear gave rise to two such T-25 cultures. Thus all cultures were initiated with approximately equal amounts of starting material. Cultures initiated in this manner reached confluency in 1-2 weeks and were used within one week thereafter.

Occasional cultures were checked for mycoplasma by the uridine phosphorylase method of Levine.[108] Although they were found to have high levels of this enzyme, the activity was resistant to treatment of the cells with heat[109] or chlorotetracycline.[110] Therefore, the cells were judged to be free of mycoplasma contamination.[108]

Growth, Labeling, And Treatment Of Cells

At the beginning of each experiment a confluent T-25 culture was trypsinized and the cells transferred to a T-75 (75 cm^2 growth area) plastic tissue culture flask. A final volume of 20 ml MEM + 5% FCS + 5% calf serum (CS) was added, and ^{14}C thymidine (Tdr) was then added to a final level of 1.25 nCi/ml. The following day the medium was replaced with 20 ml MEM + 5% FCS + 5% CS and ^{14}C Tdr added again to the same level. On the third day the medium was pipetted from the flask, the cell monolayer washed with 10 ml fresh medium, and 20 ml MEM + 5% FCS + 5% CS + 10^{-6} M Tdr was added. On the fourth day the medium was removed, and the cells were trypsinized and transferred in equal portions to fourteen T-25 flasks in 10 ml MEM + 5% FCS + 5% CS + 10^{-6} M Tdr. On the fifth and sixth days the medium was removed from the flasks and replaced with 10 ml fresh MEM + 5% FCS + 5% CS + 10^{-6} M Tdr. This segment of the protocol is illustrated in the upper portion of figure 4. Overall, during these experiments the cells were labeled with ^{14}C Tdr for two days (approximately two generation times) and then chased with 10^{-6} M Tdr for four days.

The lower portion of figure 4 illustrates the timing of the treatments given to the cells relative to the ^{3}H Tdr pulse labeling and harvest at the end of the treatment period. Specifically, the chase medium was transferred from the growth flask to a new T-25, and the treatment medium (see below) was added to the growth flask. (All chemical treatments were given in MEM without serum to avoid reaction with serum components which might create uncontrollable variation in the effective dose reaching the cells.)[111]

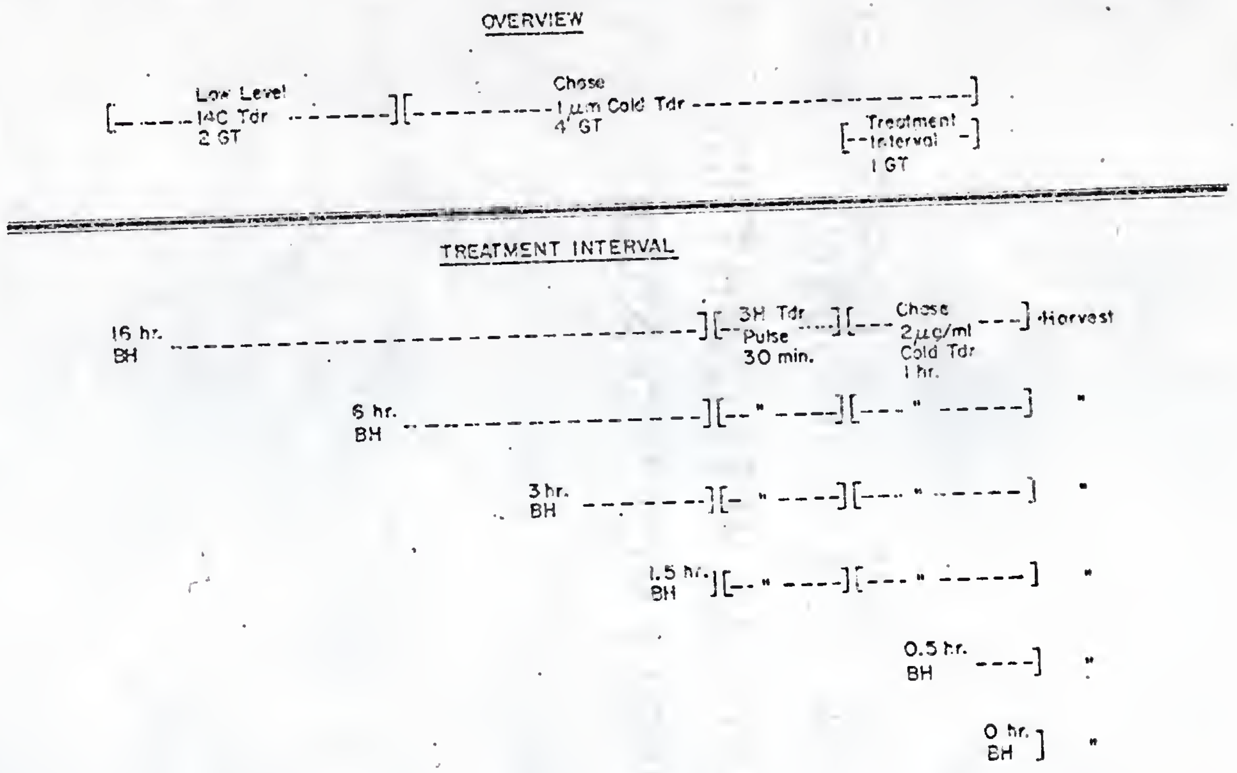


Figure 4. Protocol for alkaline elution experiments. The upper portion illustrates the labeling of parental DNA with 14C Tdr and the subsequent period of four days chase with 10^{-6} M Tdr. The lower portion illustrates the sequence during the last 16 hours for chemical treatment of the cells and the 3H Tdr pulse and one hour chase at the end of the experiments. All cultures within an experiment were harvested at the same time.

Both flasks were then placed in a 37°C , humidified incubator (90% air + 10% CO_2) for 15 minutes. The treatment medium was then removed from the cells, and the conditioned medium transferred back to the growth flask for further growth and chase as needed.

Treatment media were prepared as follows. Ethylnitrosourea was freshly dissolved before use in 0.127 M Na_2HPO_4 + 0.0369 M citrate, pH 6.0 (pH 6 buffer).[112] One milliliter of this solution was added to 9 ml MEM to give 200 micrograms/ml final concentration. Acetoxy-acetylaminofluorene was dissolved in anhydrous dimethylsulfoxide (DMSO), and aliquots were stored at -20°C. Five microliters of this solution was added to 10 ml MEM to give a 4 micromolar solution of N-AAAF. Benzo(a)pyrene-anti-diol-epoxide was dissolved in anhydrous DMSO, and aliquots were stored at -20°C. Ten microliters of this solution were added to 5 ml MEM to give a 1.35 micromolar solution of BPADE. For sham-treatment, control experiments, pH 6 buffer or DMSO was added to MEM at the appropriate concentrations.

The first treatment was given the evening of the sixth day and ended 16 hours before harvest on the seventh day. All treatments were given in duplicate. One and a half hours before harvest ^{3}H Tdr was added to all flasks that had received treatment up to that time and to the untreated, control flasks at a final level of 0.2 microcuries/ml. (The levels of ^{14}C Tdr and ^{3}H Tdr were chosen so as to minimize toxicity due to radiation damage but yet provide adequate levels for accurate counting of samples.) After 30 minutes the medium was removed from all flasks, and 10 ml of fresh MEM + 5% FCS + 5% CS + 2 micrograms/ml Tdr were added.

One hour was allotted for this final chase period. During this time two sets of flasks that had not received ^{3}H Tdr were given treatment medium for 15 minutes as described. These treatments, ending at 0.5 and zero hours before harvest, therefore received 45 minutes chase at the end of the experiments instead of one hour. At the end of the treatment period all cultures were approximately 90% confluent. The overall features of this protocol were similar to that used by Cerutti et al..[113]

The harvest was accomplished as follows. The culture flasks were taken from the 37°C incubator and placed immediately on crushed ice (cell side down). Medium was pipetted from the flasks, and 1 ml of ice cold trypsin solution (0.1% in versene) was added. Trypsinization was carried out over a five minute interval on ice by slowly rocking the flasks in order to evenly distribute the trypsin solution over the cell monolayers. The flasks were then tapped on the side to completely detach the cells, and 5-10 ml ice cold PBS was added to dilute the trypsin.

Cell Lysis

The cells were immediately poured onto 2 micron pore polyvinylchloride filters (Millipore Corp.) that had been previously cooled by addition of 2 ml ice cold PBS. (Each filter was contained in a modified Gelman, filter, vacuum funnel apparatus.) The PBS was allowed to drain through the filter, room lights were turned off, and the cells were lysed on the filter by addition of 5 ml of 0.2% Sarkosyl + 2 M NaCl + 0.02 M EDTA, pH 10. After the lysis solution drained from the filters by gravity they were washed with 3 ml of 0.02 M EDTA, pH 10. The outflow tubes of the filter funnels were then connected to a peristaltic pump, and the funnels were covered to exclude all light. A solution of 0.04 M H_4EDTA + sufficient tetrapropylammonium hydroxide to make pH 11.85 was then pumped through the filters at 0.05 ml/min and 10 fractions collected at 90 minute intervals each. All operations after and including cell lysis were carried out in subdued or excluded light to prevent fluorescent light-induced scissions of DNA which occur under alkaline conditions.[83]

Sample Collection And Counting

The 10 elution fractions were mixed with 13.5 ml Aquasol (New England Nuclear) containing 0.3%(v/v) glacial acetic acid for counting. (Glacial acetic acid was added to minimize alkali-induced chemiluminescence in Aquasol.)[83] The filters were transferred to scintillation vials, 0.4 ml of 1 N HCl were added, and the vials were capped and incubated at 70°C. for one hour. After cooling to room temperature, 2.5 ml of 0.4 N NaOH were added, and the vials were allowed to sit at room temperature for 30 minutes before adding 13.5 ml Aquasol containing 0.3%(v/v) glacial acetic acid. Filter funnels together with connecting and peristaltic pump tubing were washed by pumping through 16 ml of 0.4 N NaOH in 4 ml fractions. To the wash fractions were added 0.5 ml glacial acetic acid, to minimize the additional chemiluminescence found in these samples, and 13.5 ml of Aquasol containing 0.3%(v/v) glacial acetic acid. All samples were counted in plastic scintillation vials in a Beckman LS 8000 Liquid Scintillation System. The cell lysis, elution, and sample preparation procedures, except for minor modifications, were essentially those developed by Kohn et al..[83-84]

Alkali-Labile Site Experiments

To test for alkali-labile sites, cells were labeled, grown, and lysed on filters, and the filters washed with 0.02 M EDTA, pH 10, exactly as described above. When treatment was with ENU, the filters were then treated with ENU in pH 6 buffer for three minutes. This solution was allowed to drain under gravity from the filters which were then washed with 0.02 M EDTA, pH 10, and eluted as usual. When treatment was with N-AAAF or BPADE, the filters were washed with PBS after the EDTA wash, treated with N-AAAF or BPADE for 3 minutes, washed with PBS, then washed again with 0.02 M EDTA, pH 10, and then eluted as usual. The N-AAAF and BPADE were prepared as stock solutions in anhydrous DMSO. Appropriate aliquots of these stock solutions were added to PBS; the resulting solution was applied to the filters. Appropriate sham-treatments with pH 6 buffer or DMSO in PBS were included in the respective experiments.

Data Reduction

After backgound subtraction by the scintillation counter, correction for overlap of ^{3}H counts into the ^{14}C channel and vice versa and conversion of counts per minute to disintegrations per minute was accomplished by a Wang 600 program written by the author. The program also determined the total ^{14}C and ^{3}H disintegrations per minute (DPM) contained in the 10 elution fractions plus the filter digest plus the four funnel wash fractions and, using this, calculated the fraction of the total ^{14}C or ^{3}H DPM remaining on the filter after the collection of each of the 10 fractions.

The fraction of the total ^{14}C or ^{3}H DPM remaining after each fraction versus fraction number was plotted as in figure 5. From plots such as this, the fraction ^{14}C DPM remaining after 10.5 hours elution (sample #7) was used to calculate the fraction ^{14}C DPM eluted at that time. Also, a line tangent to the ^{3}H DPM curve was drawn to intercept the ordinate as shown. From this intercept the fraction of ^{3}H DPM not rapidly eluted was determined, and this was used to calculate the fraction of ^{3}H DPM rapidly eluted from the filter. As mentioned, the ^{14}C labeled DNA represents parental strand DNA while the ^{3}H labeled DNA represents newly synthesized DNA strands.[83,84]

The fraction eluted DNA for each culture within an experiment was plotted as the ratio of the experimental over the control values for fraction eluted. As stated above, untreated, control cultures were used in each experiment. This ratio method allows a normalization for the baseline, systematic variation between experiments and also produces some increase in sensitivity.

Tests for statistically significant differences employed the Mann Whitney U statistic.[114] The only assumptions required for proper use of this statistic are that the sample sets being compared should be randomly drawn from populations having the same, continuous distribution. This is a reasonable assumption for this application since the experiments for young and old donor cells were all performed in identical fashion and since the data were collected and manipulated in the same manner.

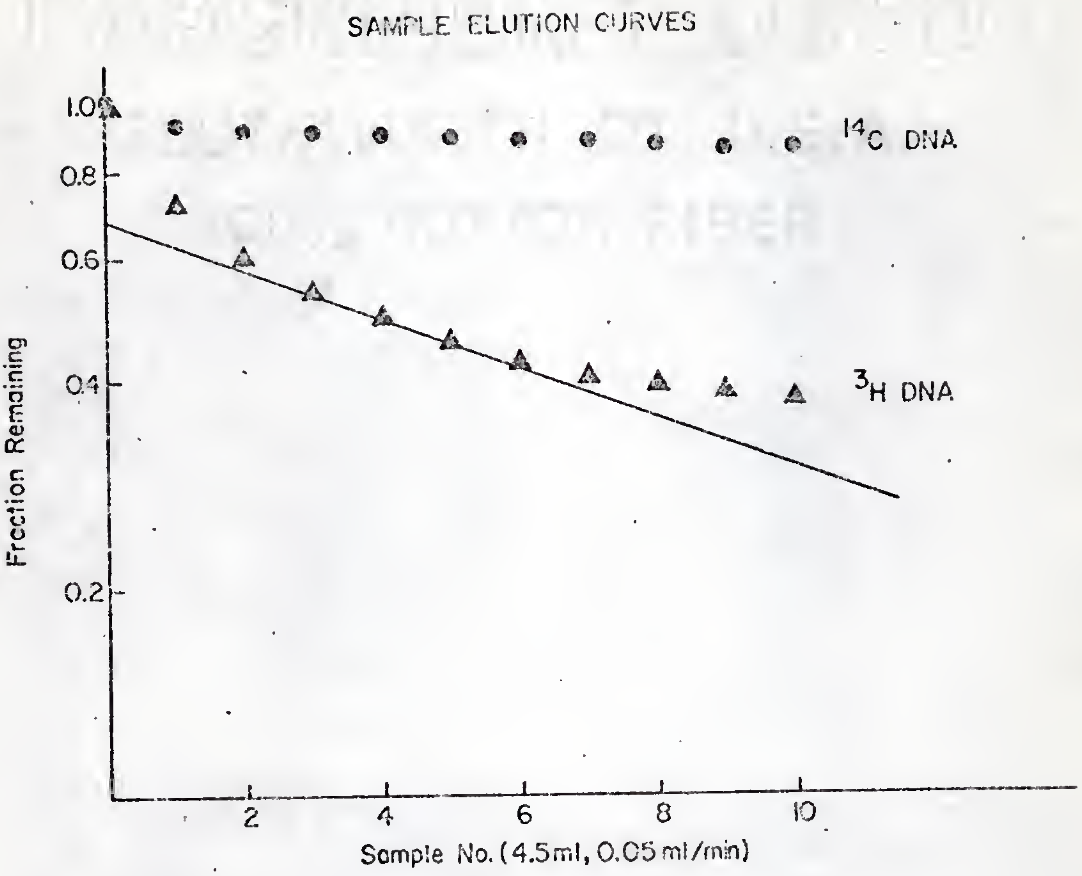


Figure 5. Sample elution curves. The fractions of the total 14C or 3H DPM remaining on the filter after the elution of each fraction are plotted on a logarithmic scale versus sample number. A line tangent to the 3H DPM curve is drawn to intercept the ordinate. The intercept value is used to calculate the fraction of the total 3H DNA that is rapidly eluted. The fraction of 14C DPM eluted after collection of sample number 7, after 10.5 hours of elution, is used as a measure of the degree of fragmentation of 14C DNA.

Chemicals

Collagenase (190 or 199 U/mg, type II) and trypsin (205, 212, or 215 U/mg) were purchased from Worthington Biochemicals. Tetrapropylammonium hydroxide was purchased from RSA Corp., Ardsley, N.Y. or Eastman Kodak. Sarkosyl was obtained as a 30% solution from K&K Laboratories. N-ethyl-N-nitrosourea was purchased from Biochemical Laboratories, Bohemia, N.Y.. N-acetoxy-2-acetylaminofluorene was purchased from ICN Pharmaceuticals. Benzo(a)pyrene-anti-diol-epoxide was obtained from IIT Research Institute, Chicago, Illinois. All serum was purchased from Gibco. Dimethylsulfoxide prepared under N_2 was purchased from Mallinkrodt. Penicillin G was purchased from Sigma Chemical Co., Streptomycin was purchased from ICN Pharmaceuticals. Schwarz-Mann supplied (^{3}H Me)-thymidine (55 Ci/mmole), and 2-^{14}C-thymidine (56 mCi/mmole) was purchased from Amersham.

CHAPTER 4
RESULTS AND INTERPRETATION

Treatment of cells with ENU leads to fragmentation of parental DNA as seen in figure 6. As stated in the Methods section, results are plotted as the ratio of the fraction DNA eluted in the untreated, control cultures for each individual experiment versus time after treatment. A ratio of one on the ordinate therefore represents the control value. Ratios greater than one indicate fragmentation of DNA above the level found in untreated cultures. Curves for fibroblasts from both three week old and two year old rats are shown here. At earlier times after treatment parental DNA is fragmented at over twice the level of the untreated cells and approaches control levels as time passes after treatment. Two phases can be distinguished in this return towards normal elutability. An early, rapid phase ending about 3 hours after treatment and a slower phase thereafter. Young and old donor cells did not differ in the earlier phase, but statistically significant differences were found at 6 and 16 hours after treatment during the slow phase. The differences between the means were 28% and 38% of the control value for 16 and 6 hours after the end of treatment, respectively. As seen, the old donor cells exhibit less fragmentation of parental DNA at later times after treatment than do young donor cells.

To interpret these results, consider that the degree of fragmentation of parent DNA seen at any given time point is the average

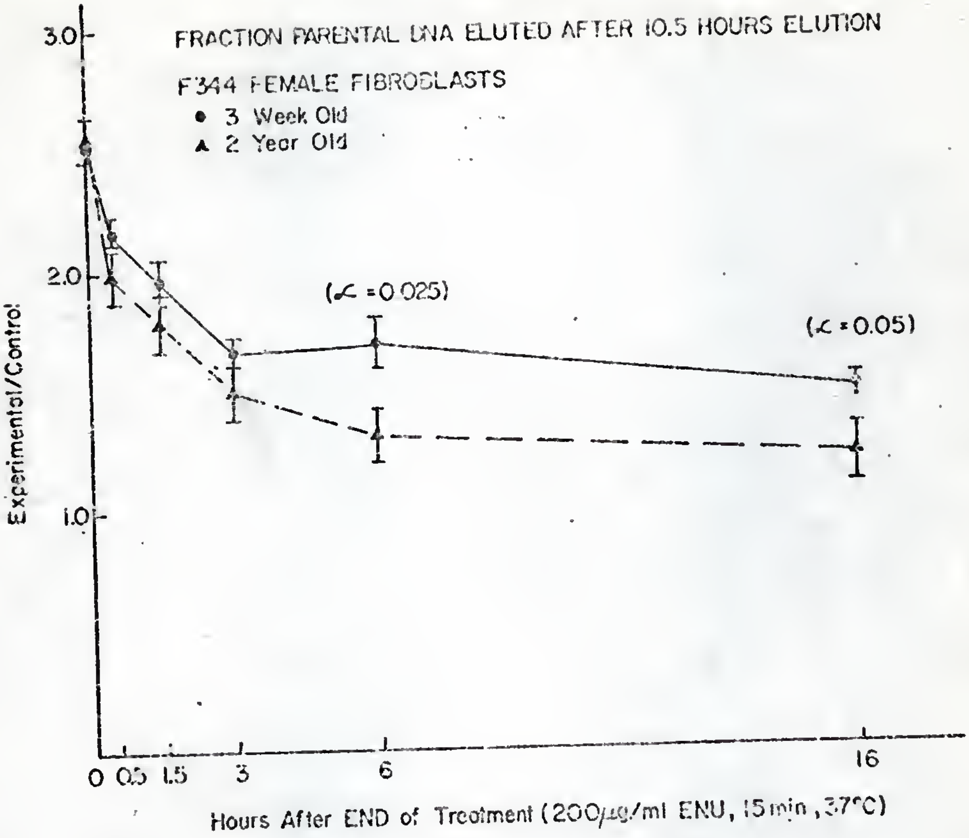


Figure 6. Normalized fraction of parental DNA eluted after 10.5 hours of elution versus time after treatment with ENU. The ratio of the fraction of parental DNA eluted in treated cultures to that eluted in untreated cultures is plotted on the ordinate. A ratio of one on the ordinate represents the degree of fragmentation found in untreated, control cultures. Points represent means ± standard error (n=10 for all points except the 6, 3, 1.5, and 0.5 hour time points for 2 year old rats where n=9). At alpha=0.05, the difference is significant at the 95% confidence level. All tests for significance employed the Mann Whitney U statistic.

sum of the fragmentation and resealing processes occurring in a culture up to that time. This occurs during the prereplication, excision repair process which involves endonucleolytic incision of the DNA adjacent to the damaged site, exonucleolytic removal of the damaged region, replacement with new DNA by DNA polymerase, and resealing by the action of DNA ligase.[55] It is the overall sum of the incision and ligation events that are represented in these curves. A higher degree of overall fragmentation indicates relatively greater incision activity than ligation activity. Greater fragmentation of parental DNA at later times in young donor cell cultures may, therefore, indicate higher residual endonucleolytic activity. This would be consistent with greater repair activity, at later times after treatment, in young donor cells than in old donor cells. It is important to note that, based on published data for human cells,[115] it is expected that more than half of the ENU-induced DNA adducts still remain in the cells used here at 16 hours after treatment.

Biphasic kinetics of DNA repair as seen here have been observed in a number of studies.[65,103,116-121] The two phases may represent repair of damage located in physically different regions of the genome. For example, damage located within nucleosomes may be less accessible to repair enzymes than that located in linker regions. Experimental evidence for this has been observed in some systems[61-64] but not in others[65,122] as mentioned in the introduction. The rapid phase could represent repair of damage in the more accessible, linker regions while the slow phase could represent repair of damage in nucleosomal core DNA. The two phases may represent repair of damage in euchromatin and heterochromatin, respectively. It may be that damage located in heterochromatic, untranscribed regions of the genome are less accessible to repair enzymes. Experimental evidence for this was mentioned earlier.[66-67] Alternatively, the two phases may represent repair of different species of DNA damage. As seen in figures 1 and 2 both N-AAAF and, especially, ENU create a variety of DNA adducts.

As noted earlier, 70% of the alkylation of DNA by ENU is on phosphate groups. Phosphotriesters thus produced are alkali-labile.[123] The $t_{1/2}$ for hydrolysis of phosphotriesters at pH 12.6 is one hour.[123] Since elution of DNA from the filters is carried out under alkaline conditions, alkali-catalyzed scission of phosphotriesters could account for part of the observed fragmentation. Figure 7 shows the results of an experiment carried out to see if significant fragmentation of ENU-treated DNA would occur under the conditions used here. In this experiment cells were labeled with ^{14}C Tdr, lysed on filters, and the DNA treated with ENU as described in Methods and Materials. Significant fragmentation of DNA did occur which increased as the concentration of ENU was increased. Although the range of concentrations used here includes that used in treating cells (200 micrograms/ml), no direct comparison can be made as to the levels of damage incurred in the two types of experiments. In the experiment of figure 7 naked DNA was treated with ENU, while in the experiments of figure 6 whole cells were treated. The effective concentration of ENU within the whole cells is expected to be less than that when naked DNA was treated with the same concentration of ENU. As mentioned earlier, ethylphosphotriesters are not known to be removed from cellular DNA.[88-89]

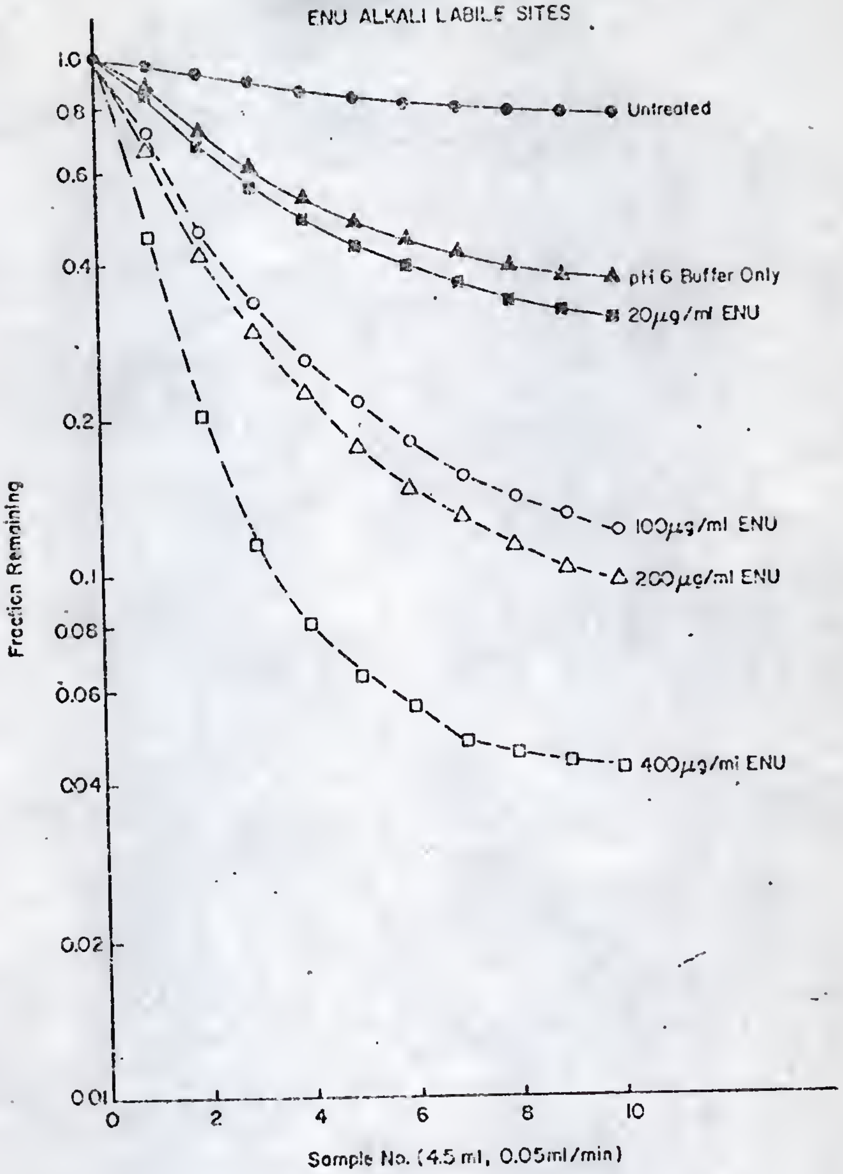


Figure 7. Log of the fraction DNA remaining on the filters after collection of each sample versus sample number. Naked DNA on the filters was treated with ENU as described in Methods and Materials. Points represent the means of duplicates.

Therefore, alkali-induced scission cannot account for the decreasing fragmentation seen in figure 6. However, if the experiments were carried out over a longer post-treatment incubation period, one might predict that the curves for both young and old donor cells would eventually level off at the same degree of fragmentation. This level would be higher than that for the untreated controls and would be due to persistent ethyl phosphotriesters.

Since ENU was given to the cells in medium devoid of serum it was necessary to do an experiment in which the cells were treated with medium only. Since the ENU was dissolved in a pH 6 buffer, as described earlier, the appropriate amount of this buffer was added in this experiment. The results for parent strand fragmentation are shown in figure 8. As can be seen, the effect is negligible compared to that obtained with ENU.

The effect of ENU on daughter strand elongation can be seen in figure 9. The old donor cells exhibited greater elongation of daughter strand DNA which essentially reached the length found in untreated, control cultures by six hours after treatment. Daughter strand DNA in young donor cells did not reach control levels during the course of the experiments. Statistically significant differences were found between all pairs of time points as indicated in the figure. The differences were 16%, 31%, 23%, and 21% of control values at 10, 6, 3, and 1.5 hours after treatment, respectively.

The rate of elongation of DNA synthesized within a 1.5 hour time block which commences at various times after treatment is being measured here. This DNA is synthesized opposite a previously

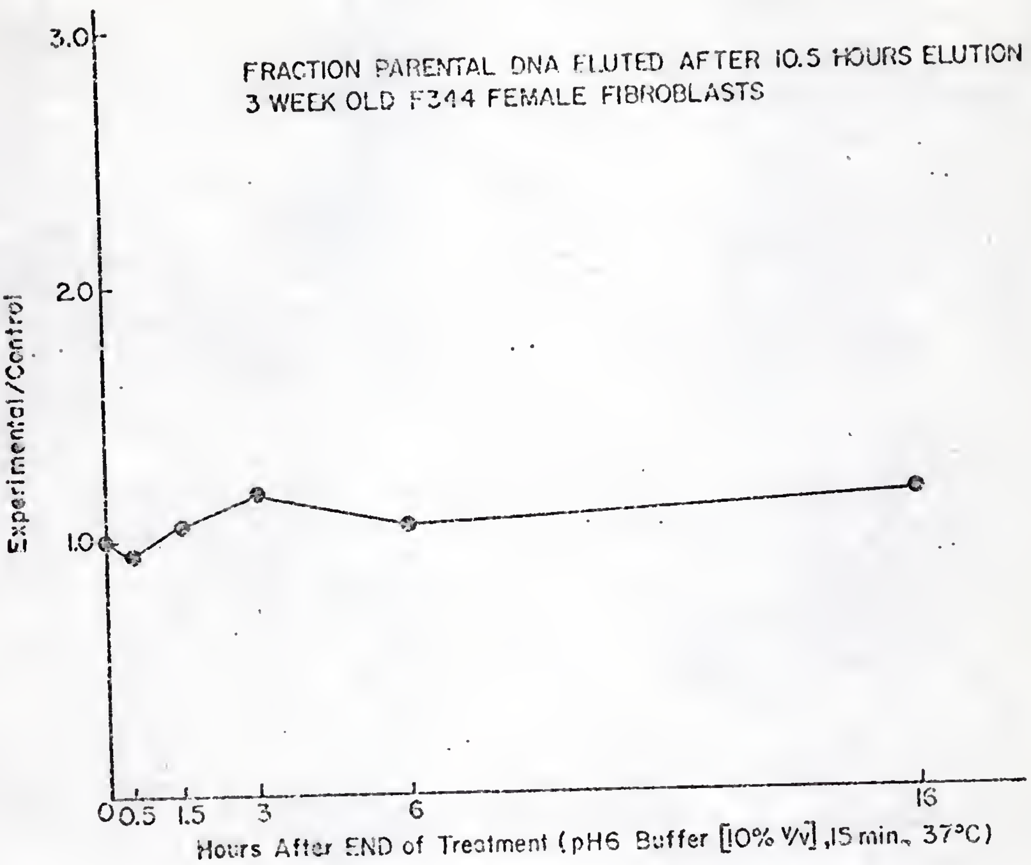


Figure 8. Relative fragmentation of parental DNA after sham-treatment with the pH 6 buffer (see Methods and Materials) used in the ENU experiments. Results are plotted as in figure 6. Points represent the means of duplicates.

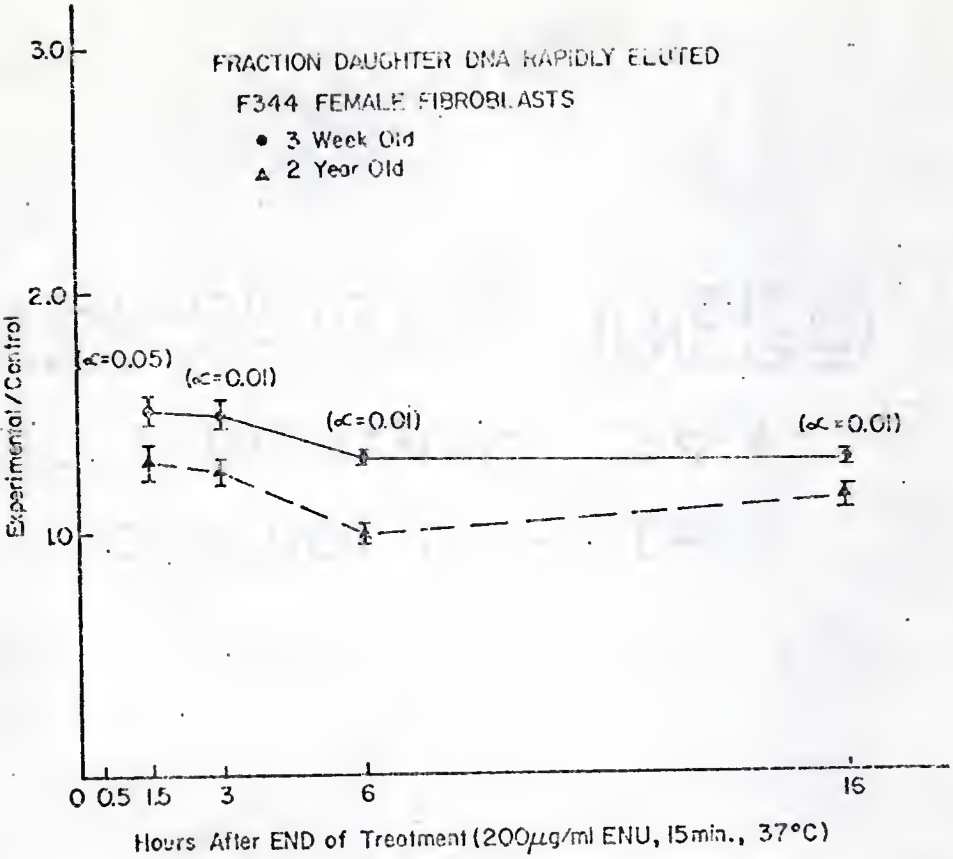


Figure 9. Normalized fraction of undamaged, newly synthesized, daughter strand DNA rapidly eluted versus time after treatment with ENU. The ratio of the fraction of daughter strand DNA rapidly eluted in treated cultures to that rapidly eluted in untreated, control cultures is plotted on the ordinate. A ratio of one on the ordinate thus represents the amount of rapidly eluted, daughter DNA found in untreated, control cultures. Points represent means ± standard error (n=10 for all points except the 6, 3, and 1.5 hour time points for 2 year old rats where n=9). Statistical analysis was as described for figure 6.

damaged template. If damage remains in the DNA it could create a block to the elongation of daughter strands. If the parental strand damage were removed or if the DNA replication machinery were able to bypass the damaged region of the template, no block to daughter strand elongation would occur.

Replication of DNA opposite a damaged, parental template is known to occur,[124] and several possible mechanisms have been proposed.[125] One is a recombination type mechanism where a segment of the daughter strand of one duplex and a corresponding section of the damaged strand exchange places so that replication may continue. The result of this would be the appearance of damaged residues in untreated, daughter strand DNA. Experimental evidence for this in mammalian cells has been presented.[126-127] However, it has been argued that those results are also consistent with bypass of template damage without the need to postulate recombinational events.[128]

This could be accomplished if bases were inserted in random fashion opposite the damaged region merely to get on with replication. This would result in the introduction of inappropriate bases in the daughter strand and would, therefore, increase the error level of the cell. It would be analogous to "SOS," error prone postreplication repair in bacteria.[129] Some evidence for an inducible, error prone DNA repair in mammalian cells has also been presented.[130] Another bypass mechanism would be for the daughter strand of one duplex, having replicated past the region on its parent strand corresponding to the damaged region on the other parent strand, to fold back at the replication fork and base pair with the daughter strand of the damaged duplex. Replication could thus continue on both daughter strands as far as it had on the first one thereby bypassing the damaged region.[131] These three mechanisms are all possible pathways of postreplication repair (see Discussion).

If one accepts that the young donor cells are more active at removing parent strand damage (figure 6), then one possible interpretation would be that the old donor cell replication machinery is more adept at bypassing template damage than that of young donor cells. An alternative interpretation is that single-stranded regions formed during the excision repair process create blocks to the elongation of daughter strands. Since the young donor cells incise damaged DNA more actively (as seen in figure 6), there would be more blocks to daughter strand elongation in these cells.

Figure 10 shows the effect of sham-treatment on daughter strand DNA elongation. As can be seen there is hardly any effect at all.

Treatment of cells with DNA damaging agents in known to cause suppression of DNA synthesis.[132] It was of interest to know if the overall rate of DNA synthesis would be less in old donor cells after treatment. The ratio of the total ^{3}H DPM/total ^{14}C DPM relative to that of the untreated controls after ENU treatment is plotted on the ordinate in figure 11. (Again, a ratio of one on the ordinate indicates the untreated, control value.) Total DNA synthesis was suppressed equally in both young and old donor cells. Although there was some recovery after six hours, DNA synthesis was suppressed slightly more after 16 hours than at earlier time points.

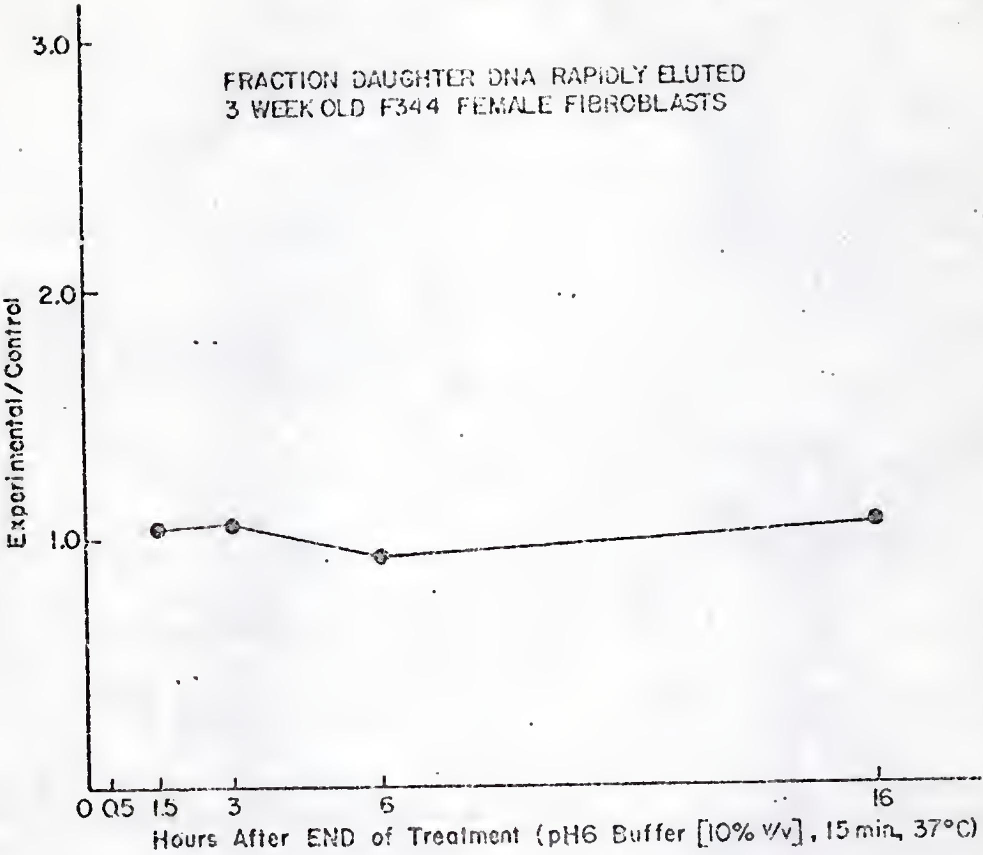


Figure 10. Relative amount of rapidly eluted, newly synthesized, daughter strand DNA found after sham-treatment with the pH 6 buffer (see Methods and Materials) used in the ENU experiments. Results are plotted as in figure 9. Points represent the means of duplicates.

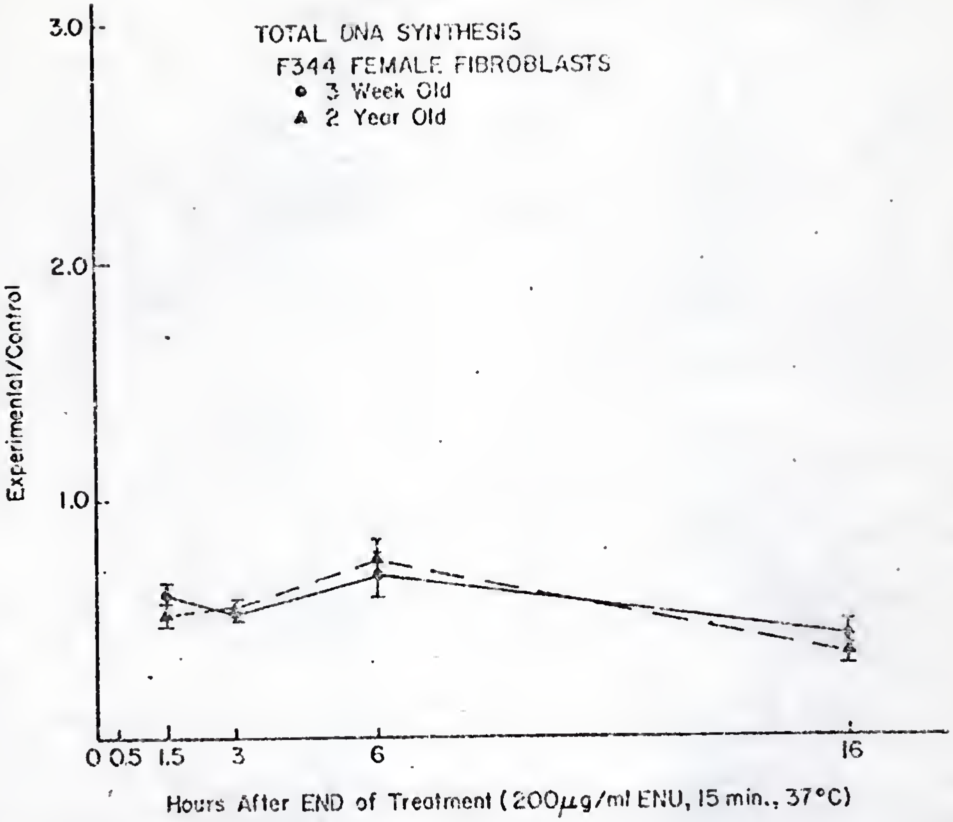


Figure 11. The ratio of the total 3H DPM/total 14C DPM in ENU-treated cultures divided by that in untreated, control cultures versus time after treatment. A ratio of one on the ordinate represents the overall rate of DNA synthesis, during the last 1.5 hours of the experiments, in untreated, control cultures. Points represent means ± standard error (n=10 for all points except the 6 and 3 hour time points for 2 year old rats where n=9). Statistical analysis was as described for figure 6.

Figure 12 shows the suppression of DNA synthesis found in sham-treated cultures. Significant suppression is seen early after treatment, but DNA synthesis returns to the level found in untreated cultures by 16 hours. This obviously was not the case with ENU-treated cultures.

Experiments using N-AAAF and BPADE were performed in essentially the same manner as those using ENU. The only difference being that N-AAAF and BPADE were dissolved in DMSO instead of pH 6 buffer before addition to MEM in the culture flasks.

Fragmentation of parental DNA after N-AAAF is seen in figure 13. The response obtained with the dose used was much greater than seen in the ENU experiments. This could partially account for the greater variance found for the young donor cell curve in figure 13. However, this variance was largely accounted for, as was the variance in all these experiments, by variation between experiments rather than variation between duplicates within a given experiment. A different primary culture was utilized to derive the cells used for each experiment. This fact alone could account for the observed variation. However, it was necessary during the course of the experiments to use different batches of media and sera which may have contributed even further variation. Nevertheless, when analyzed on a statistical basis, the differences between means were found to be significant for all time points at the levels indicated in figure 13. Differences between the means were 153%, 235%, 298%, 342%, 288%, and 461% of the untreated, control values for 16, 6, 3, 1.5, 0.5 and zero hours after the end of treatment, respectively. Greater fragmentation of parental DNA was seen at all time points

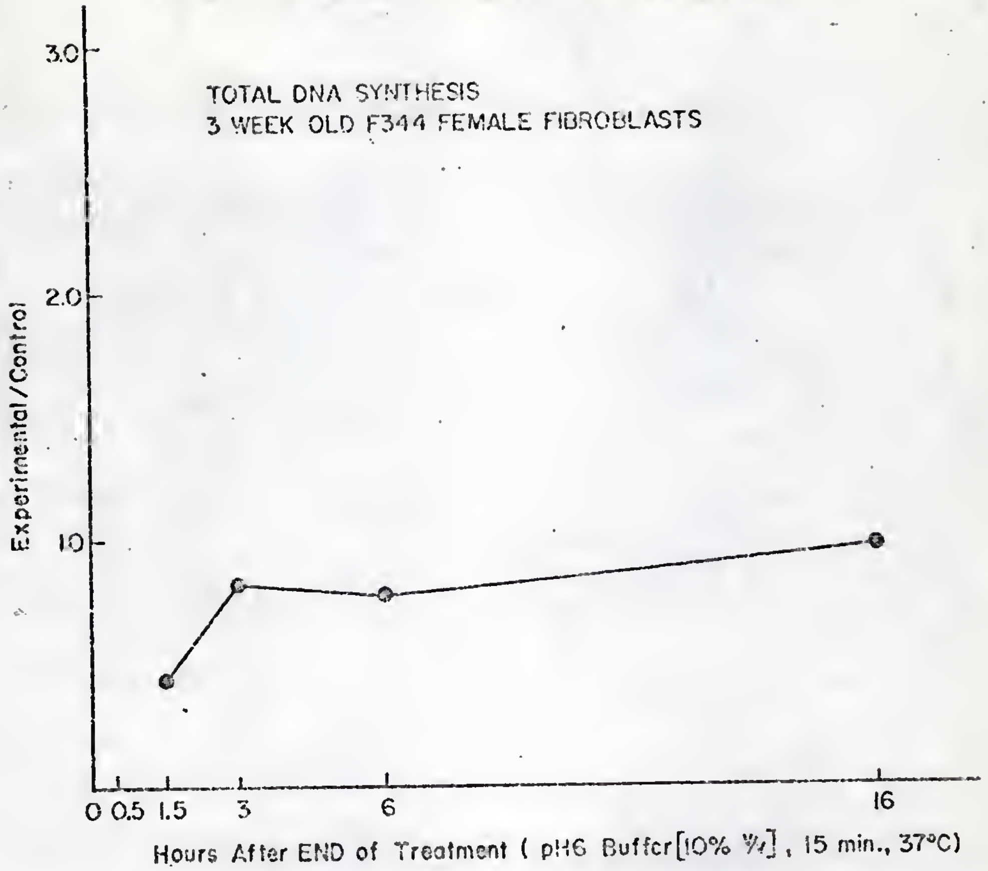


Figure 12. Relative rate of DNA synthesis during a 0.5 hour time interval at various times after sham-treatment with the pH 6 buffer (see Methods and Materials) used in the ENU experiments. Results are plotted as in figure 11. Points represent means of duplicates.

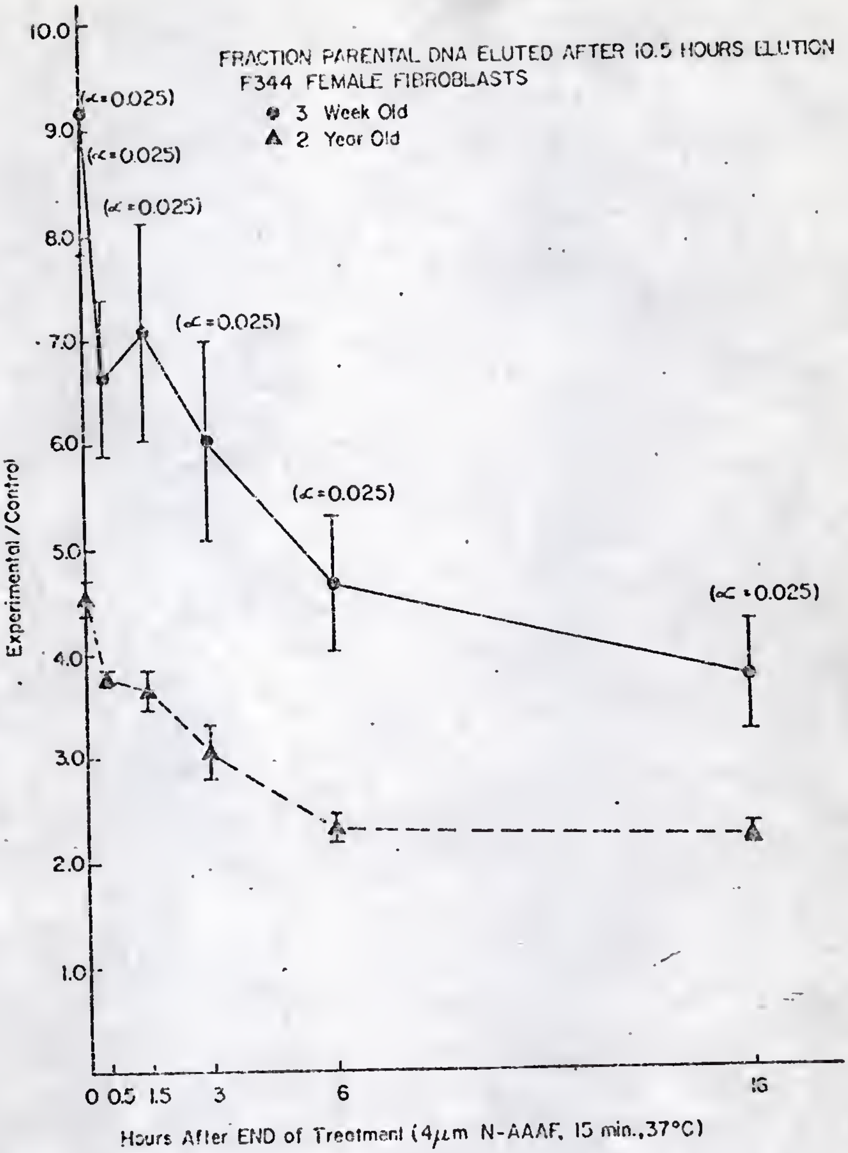


Figure 13. Normalized fraction of parental DNA eluted after 10.5 hours of elution versus time after treatment with N-AAAF. Results are plotted as in figure 6. Points represent means ± standard error (n=4). Statistical analysis was as described for figure 6.

in the young donor cells; a result very similar to that found after treatment with ENU. In these experiments the initial degree of fragmentation in young donor cells at zero hours after the end of treatment was considerably greater than in old donor cells. This was not seen in the ENU experiments. In addition, at 1.5 hours after the end of treatment there appears to be a resurgent increase in fragmentation over that seen at 0.5 hours; also seen to a lesser extent in the old donor cells. Although the magnitude of the increase is less than the standard error in either case, this trend could possibly have arisen from the greater disruption of chromatin structure expected with N-AAAF and/or, secondarily, from the much greater repair response obtained. A greater challenge to the repair systems may have allowed the finer aspects of fragmentation kinetics during the early phase to become manifest. With ENU which would create less distortion of the helix, the young and old repair systems were apparently functionally equivalent during the initial phase. Calculations based on data reported by Goth-Goldstein,[115] Singer,[86] and Amacher et al.[91] indicate the level of total adducts formed per mole of DNA phosphate by ENU to be approximately twice that formed by N-AAAF at the concentrations used in these experiments. These are only rough estimates and depend upon the assumption of a linear relation between concentration and number of adducts formed. They suggest that a much greater repair response is elicited by relatively few N-AAAF adducts when compared to ENU.

Although no reports of N-AAAF-induced alkali-labile bonds have appeared, this possibility was examined. The results of treating naked DNA on filters with N-AAAF, as described in Methods and Materials, is shown in figure 14. Increasing rate of elution of DNA from the filters is seen with increasing concentration of N-AAAF. Judging from these results, the degree of alkali-induced fragmentation of DNA after N-AAAF treatment in the experiments reported here would be much less than after ENU treatment. The effect of this fragmentation on the results obtained would be essentially as discussed for the ENU experiments.

Although some fragmentation of DNA after DMSO sham-treatment is apparent (figure 15), it is not greater than 21% of the control value and certainly does not account for the results seen with N-AAAF treatment.

Elongation of daughter strands after N-AAAF treatment (figure 16) was less than after ENU treatment; however, the results were qualitatively the same. Daughter DNA was elongated to a greater degree in old donor cells, and statistically significant differences, as indicated in figure 16, were found between all pairs of time points. Differences between the means were 81%, 51%, 102%, and 99% of control values at 16, 6, 3, and 1.5 hours after the end of treatment, respectively. Sham-treatment had essentially no effect on daughter strand elongation as seen in figure 17.

Suppression of total DNA synthesis was greater after N-AAAF (figure 18) than after ENU treatment. Statistically significant differences between the means were found for the 16 and 3 hour time points. However, these differences, 18% and 6% of the control

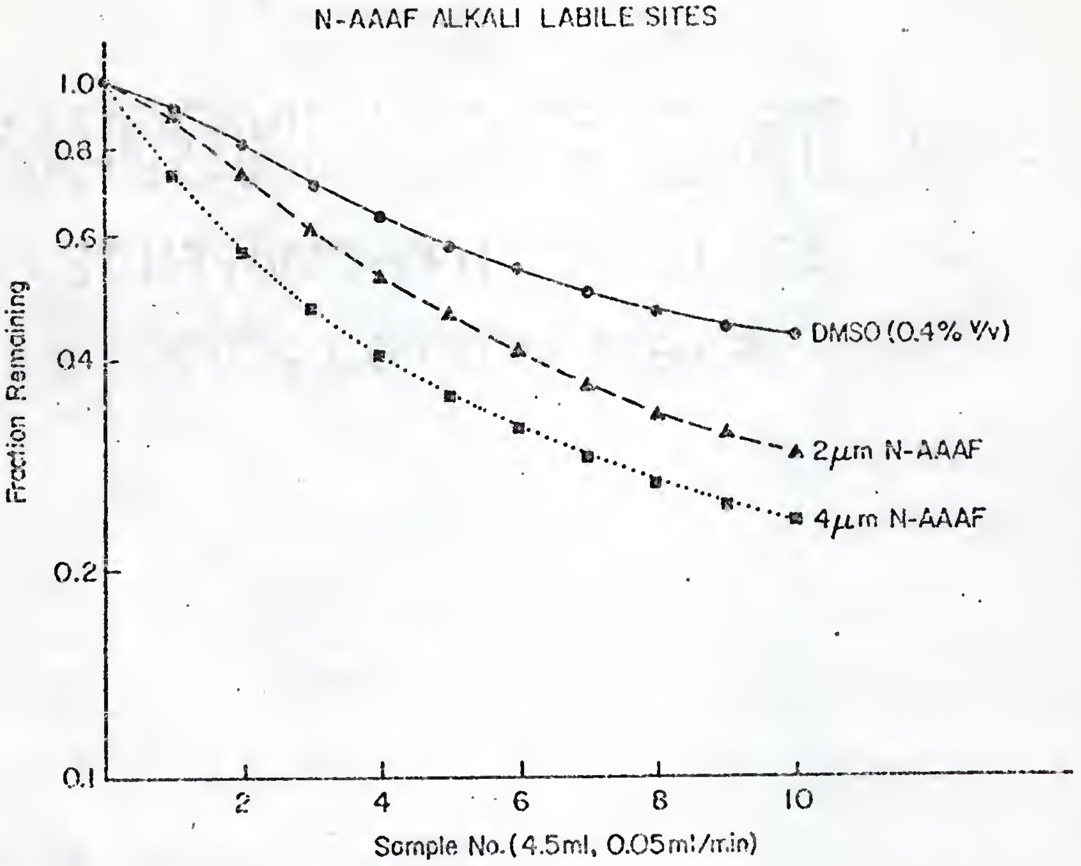
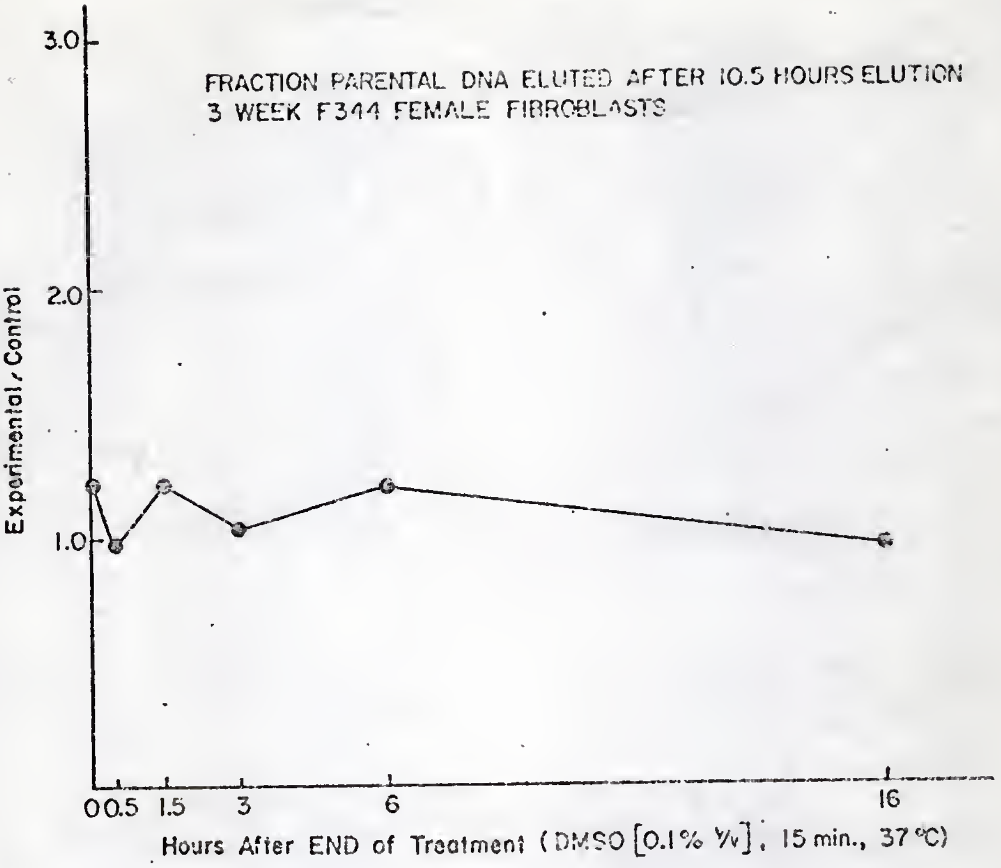


Figure 14. Log of the fraction of DNA remaining on the filters after collection of each sample versus sample number. Naked DNA on the filters was treated with N-AAAF as described in Methods and Materials. Points represent the means of duplicates.



Chemicals

Collagenase (190 or 199 U/mg, type II) and trypsin (205, 212, or 215 U/mg) were purchased from Worthington Biochemicals. Tetrapropylammonium hydroxide was purchased from RSA Corp., Ardsley, N.Y. or Eastman Kodak. Sarkosyl was obtained as a 30% solution from K&K Laboratories. N-ethyl-N-nitrosourea was purchased from Biochemical Laboratories, Bohemia, N.Y.. N-acetoxy-2-acetylaminofluorene was purchased from ICN Pharmaceuticals. Benzo(a)pyrene-anti-diol-epoxide was obtained from IIT Research Institute, Chicago, Illinois. All serum was purchased from Gibco. Dimethylsulfoxide prepared under N_2 was purchased from Mallinkrodt. Penicillin G was purchased from Sigma Chemical Co., Streptomycin was purchased from ICN Pharmaceuticals. Schwarz-Mann supplied

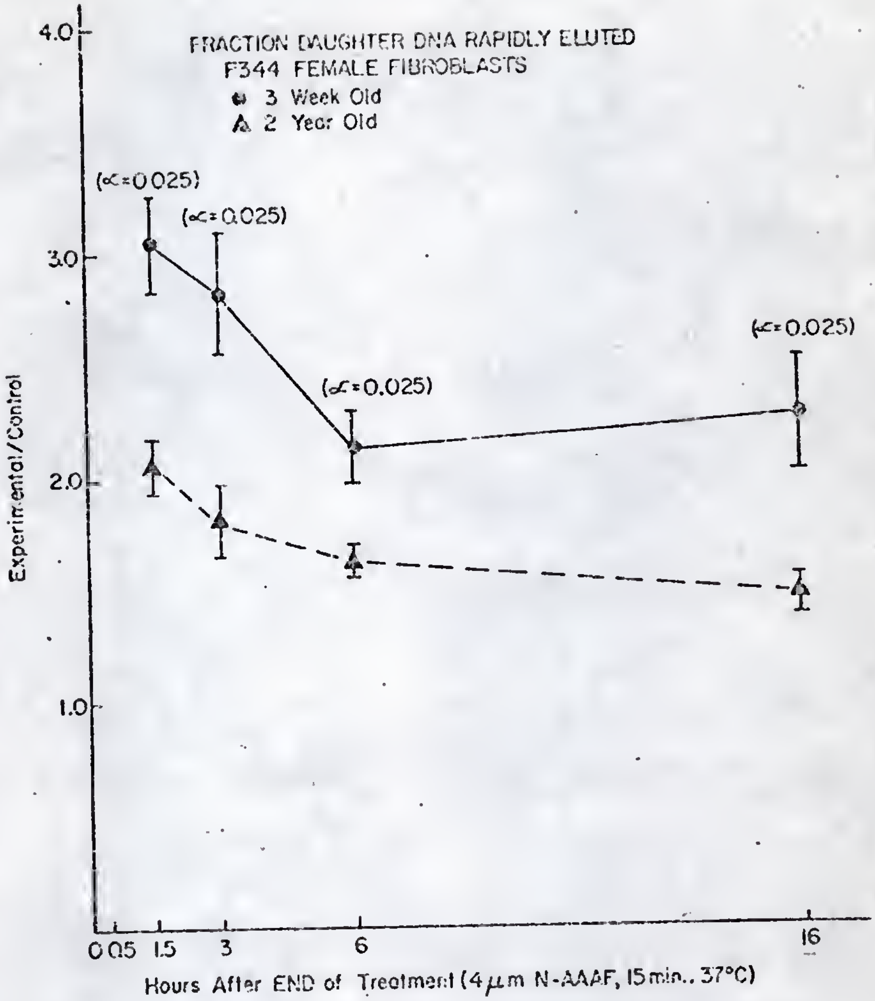


Figure 16. Normalized fraction of undamaged, newly synthesized, daughter strand DNA rapidly eluted versus time after treatment with N-AAAF. Results are plotted as in figure 9. Points represent means ± standard error (n=4). Statistical analysis was as described for figure 6.

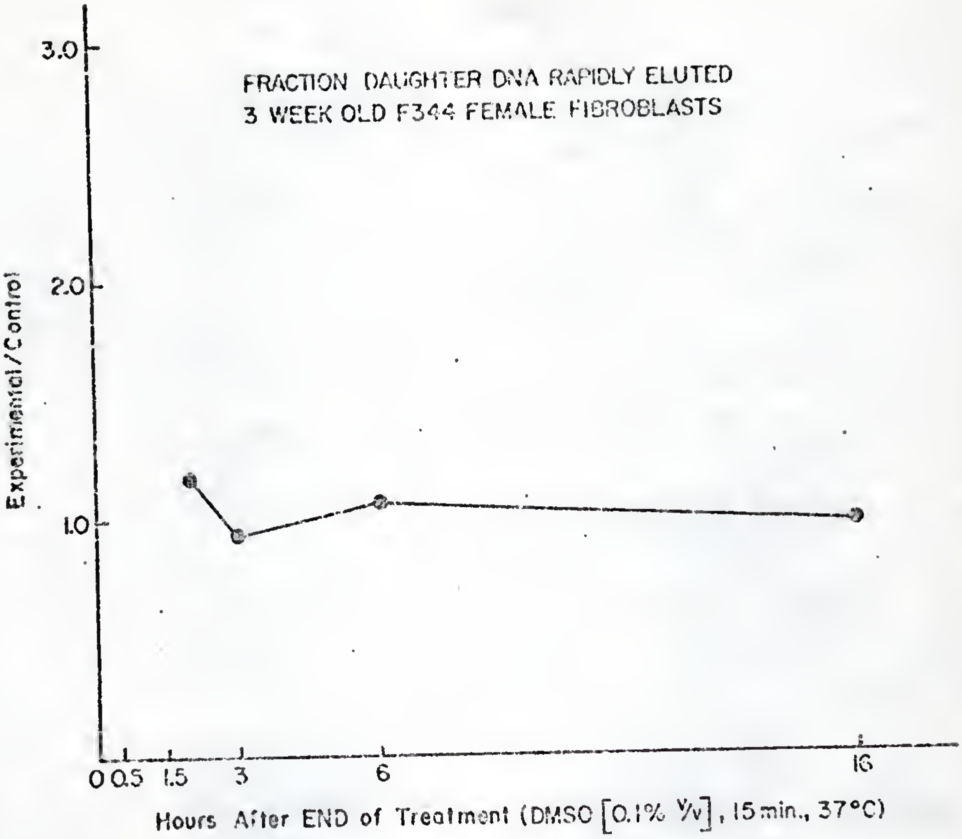


Figure 17. Relative amount of rapidly eluted, newly synthesized, daughter strand DNA found after sham-treatment with DMSO. Results are plotted as in figure 9. Points represent the means of triplicates.

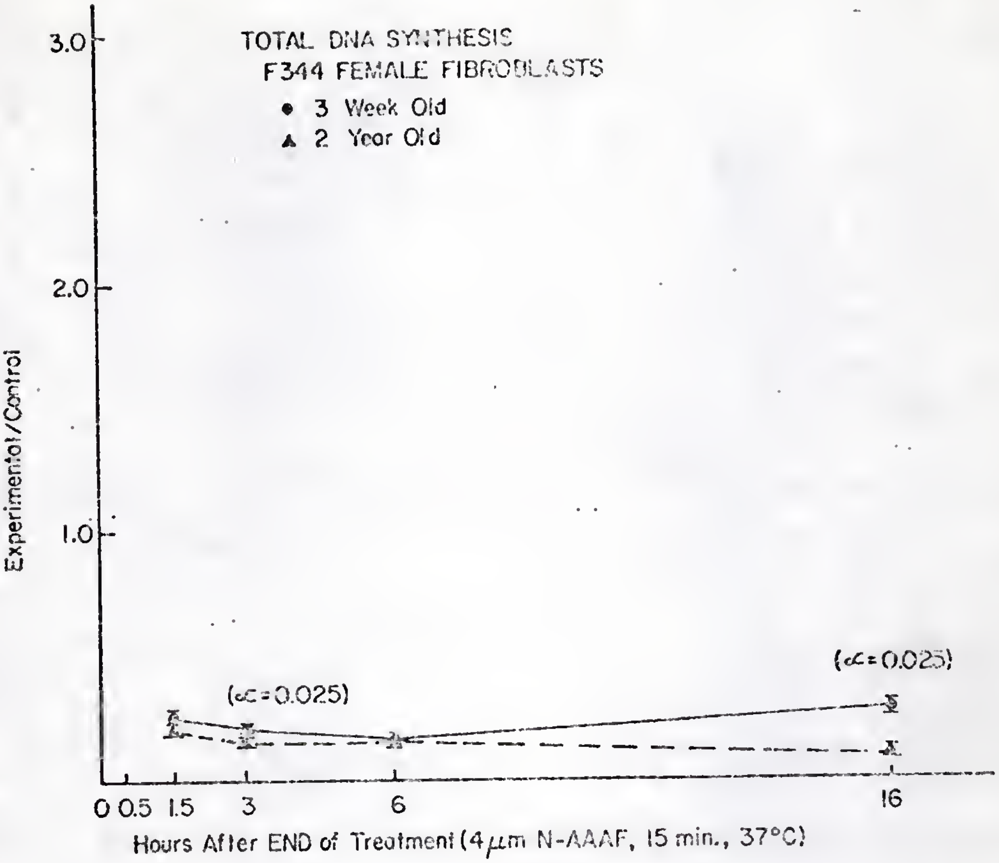


Figure 18. The ratio of total 3H DPM/total 14C DPM in N-AAAF-treated cultures divided by that in untreated, control cultures versus time after treatment. Results are plotted as in figure 11. Points represent means ± standard error (n=4). Statistical analysis was as described for figure 6.

values at 16 and 3 hours, respectively, were numerically small when compared to the other differences found after N-AAAF treatment. Sham-treatment with DMSO (figure 19) yielded a slight suppression of DNA synthesis seen during the first three hours after treatment. This result is consistent with that seen after pH 6 buffer sham-treatment (figure 12).

The total number of experiments using BPADE was not sufficient to allow statistical analysis using the Mann Whitney U statistic as in the other experiments. However, certain aspects of the response obtained after BPADE treatment appear similar to the responses after ENU and N-AAAF treatment. As seen in figure 20, parent strand fragmentation was greatest in young donor cells and virually nonexistent in old donor cells. Fragmentation in young donor cells returned to the levels found in sham-treated control experiments (figure 15) by 6 hours after the end of treatment. A trend towards resurgent fragmentation of parent strand DNA is apparent at 1.5 hours as was the case for N-AAAF.

Benzo(a)pyrene-diol-epoxides are known to form phosphotriesters in DNA with low frequency,[133-134] which are expected to lead to fragmentation of DNA under the alkaline conditions in these experiments. Figure 21 provides evidence for this. Naked DNA on filters was treated with four levels of BPADE in this experiment, the highest of which, 2.7 micromolar, led to increased fragmentation of DNA. Although a small amount of nicking of DNA at BPADE-induced phosphotriesters is expected at physiological pH,[133] the overall consequences of BPADE-induced phosphotriesters for the results reported

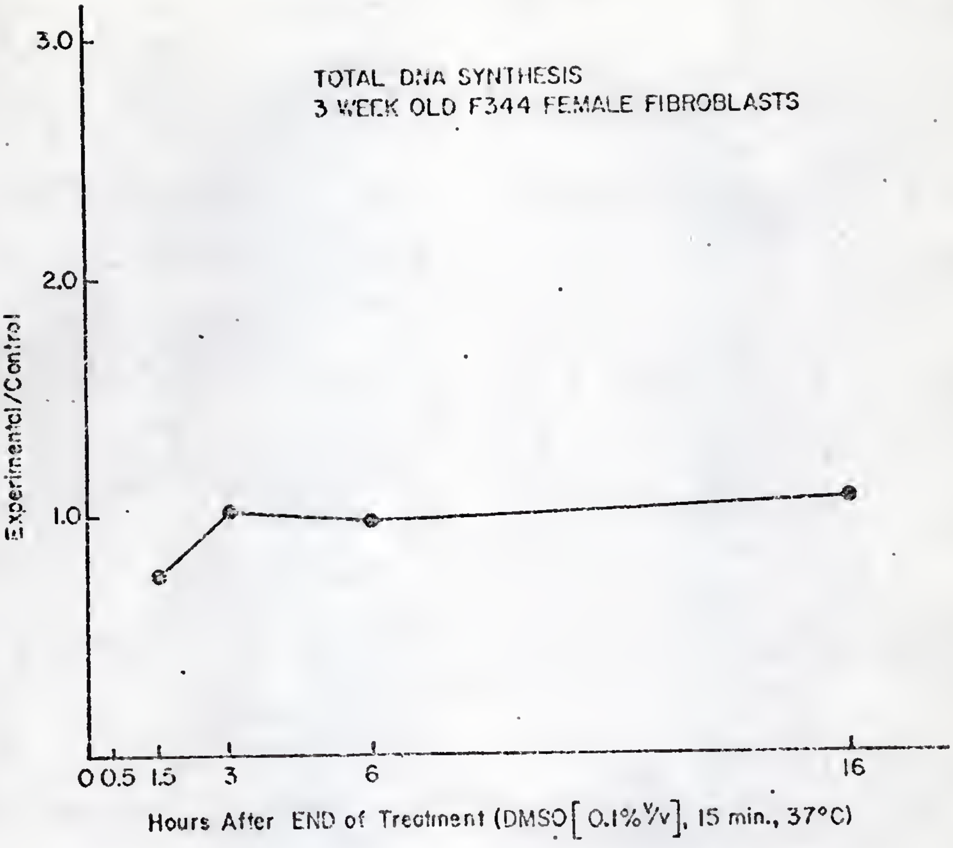


Figure 19. Relative rate of DNA synthesis during a 0.5 hour time interval at various times after sham-treatment with DMSO. Results are plotted as in figure 11. Points represent the means of triplicates.

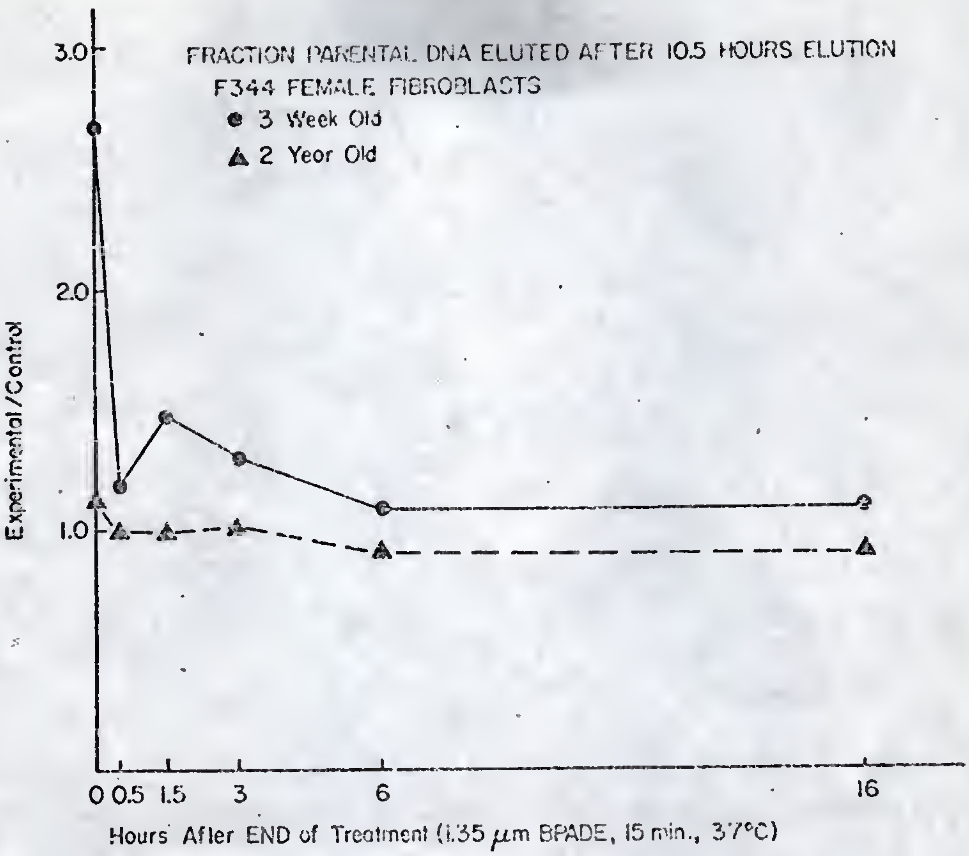


Figure 20. Normalized fraction of parental DNA eluted after 10.5 hours of elution versus time after treatment with BPADE. Results are plotted as in figure 6. Points represent means of quadruplicates (3 week old rats) or duplicates (2 year old rats).

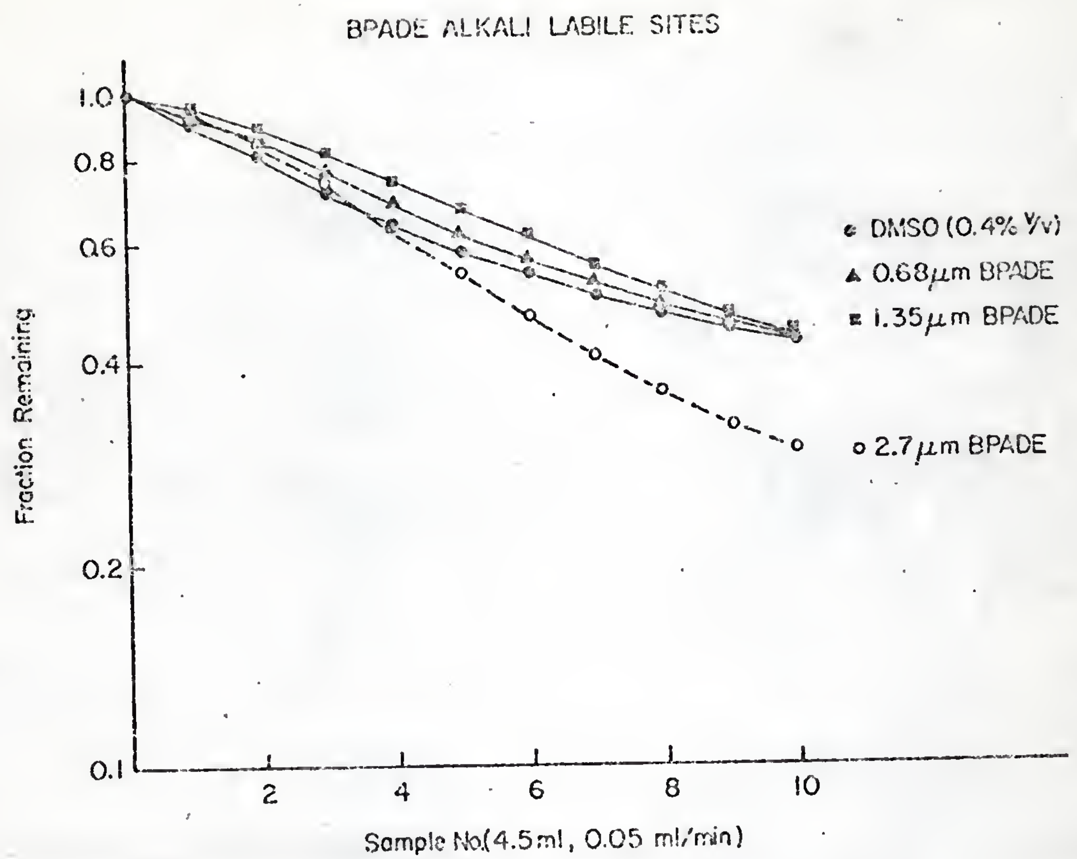


Figure 21. Log of the fraction of DNA remaining on the filters after collection of each sample versus sample number. Naked DNA on the filters was treated with BPADE as described in Methods and Materials. Points represent the means of duplicates.

here would be essentially as discussed earlier for the ENU and N-AAAF experiments.

While the curves for daughter strand elongation after BPADE treatment (figure 22) of young and old donor cells are very similar at later times after the end of treatment, a divergence is evident at the 1.5 and 3 hour time points. Less elongation is evident in the old donor cells at early times after treatment; a result converse to those after ENU and N-AAAF treatments.

Although there is suppression of DNA synthesis early after BPADE treatment (figure 23) with no recovery, as was seen after ENU and N-AAAF treatment, more suppression is apparent in the old donor cells. This was not seen in the other experiments.

Certain aspects of the response to BPADE treatment may differ from those obtained after ENU and N-AAAF treatment, but a greater number of experiments would be required in order to verify this.

The differences between young and old donor cells seen here could possibly be accounted for if there were differences in the rate of cell division and/or cell survival after treatment. If the old donor cells divided more rapidly than the young donor cells, the damaged residues might be diluted out. This would result in a lower repair response observed at the time of harvest. When the cultures were first initiated the old donor cells appeared to grow slightly slower than the young donor cells. This has been observed in other systems,[46] but is the opposite situation to that required to produce the observed difference in repair response.

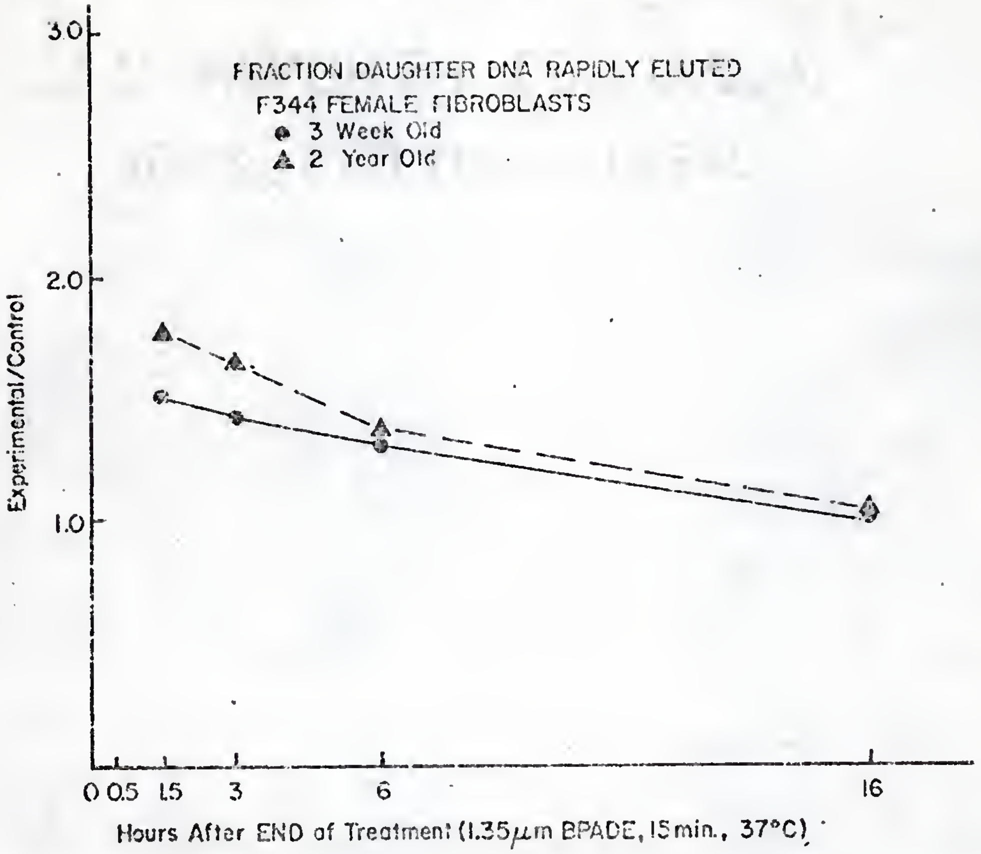


Figure 22. Normalized fraction of undamaged, newly synthesized, daughter strand DNA rapidly eluted versus time after treatment with BPADE. Results are plotted as in figure 9. Points represent means of quadruplicates (3 week old rats) or duplicates (2 year old rats).

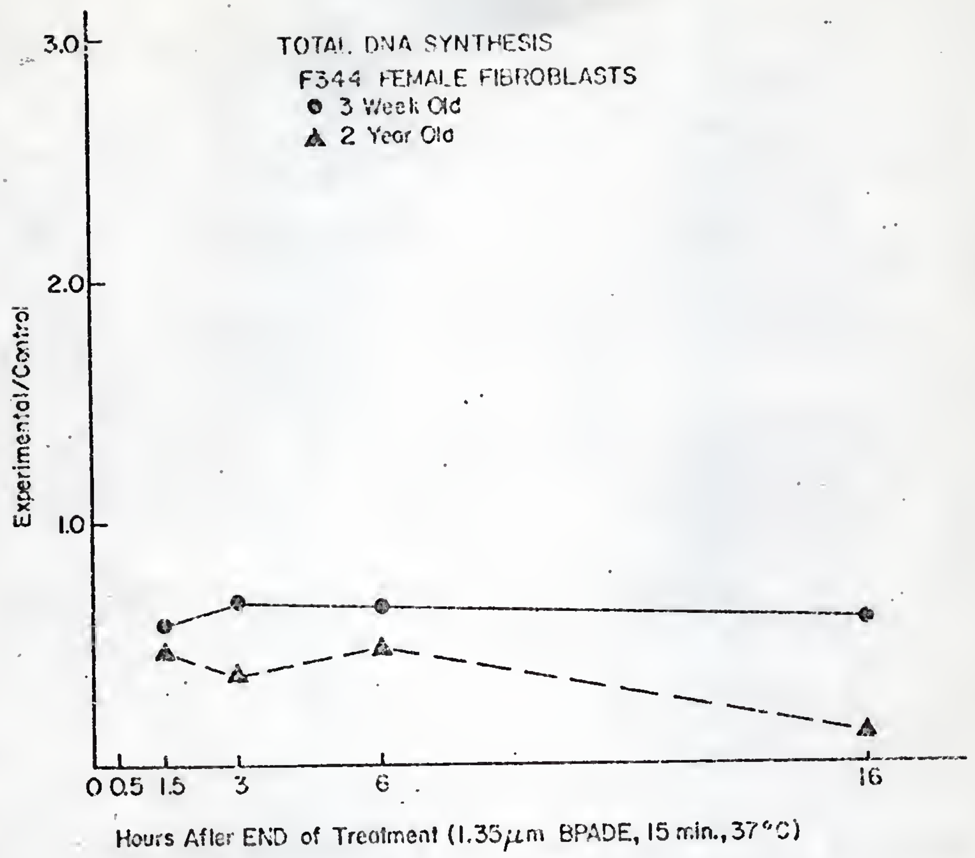


Figure 23. The ratio of total 3H DPM/total 14C DPM in BPADE-treated cultures divided by that in untreated, control cultures versus time after treatment. Results are plotted as in figure 11. Points represent means of quadruplicates (3 week old rats) or duplicates (2 year old rats).

At the end of the experiments all cultures were approximately 90% confluent for both young and old donors; therefore, any differences in growth rate were not apparent during the treatment and repair interval. Also, the overall rate of DNA synthesis at the end of the experiments in the untreated, control cultures, as measured by the ratio of total ^{3}H DNA/total ^{14}C DNA, was not significantly different between young and old cultures.

Cytotoxicity in these experiments was estimated by determining the total ^{14}C DPM in cultures incubated for 16 hours after treatment relative to that in untreated, control cultures. In all cases the loss of ^{14}C DPM was less than 10%, and there was no statistically significant difference in this loss between young and old donor cultures. However, cell killing might occur without detachment from the growth surface, and this cannot be ruled out. Greater cell killing in the old donor cultures might account for the results presented here. If this were the case it would mean that the old donor cells are less resistant to the damaging insults presented to them. Decreased overall resistance to DNA damaging agents would be an expected result of decreased DNA repair efficiency.

Differences between cultures may have resulted if equal concentrations of chemical agent in the media created different levels of DNA damage in young and old donor cultures. This cannot be entirely ruled out but is unlikely. The three chemicals used act directly, requiring no metabolic activation by the cell.[91,112,135] Differences in cell volume or macromolecular content might lead to differences in reactivity with cellular contents and, therefore, to differences in the level of adducts formed in the DNA. However, Schneider and Mitsu found that fibroblasts derived from human donors show no differences in cell volume or macromolecular content as a function of donor age.[46] If this can be assumed to be true for the rat fibroblasts used here, there is no reason to expect unequal initial DNA adduct levels between young and old donor cells.

Differential inactivation of repair or metabolic systems between young and old donor cells could have been a factor in these experiments. If equal amounts of alkylation or arylalkylation of components other than DNA created unequal impairments in metabolism and/or DNA repair, this might account for the results obtained. If substantiated, this would be a useful finding in itself. However, the fact that total DNA synthesis was suppressed essentially to the same extent by ENU and N-AAAF treatment argues against this. If preferential inactivation of old donor cell enzymes occurred, we might expect it to be manifest in the overall level of DNA synthesis as well.

One final possibility is that increased levels of protein-DNA or DNA-DNA crosslinks in old donor cells could account for the decreased rate of elution of old donor cell DNA from the filters. Apparently, DNA-protein crosslinks do increase with age in mammals.[37,44] It is also known that DNA-protein crosslinks inhibit elution of DNA from polyvinylchloride filters under alkaline conditions.[136-139] If this was involved in these experiments, one might expect DNA from untreated, control, old donor cultures to be eluted at a slower rate than that from untreated, control, young donor cultures. This was not observed.

CHAPTER 5
DISCUSSION

The results reported here provide further evidence for declining DNA repair capacity with *in vivo* aging in mammals. However, I feel this work should be extended and complemented by studies of the removal of specific adducts from young and old donor cells. Virtually every agent known to damage DNA produces several species of damage, each of which may be repaired with different efficiency and kinetics. This has been demonstrated for ENU[115] and N-AAAF.[140] In the case of ENU, O6-ethylguanine has been shown to be removed much more slowly from rat brain DNA than the other base adducts formed.[87] This correlates with preferential production of brain tumors in those rats.[141] When compared to the overall level of adducts, O6-ethylguanine is a relatively minor product,[86] yet it has the highest correlation with tumor production.[142] Likewise, repair of only certain species of DNA damage produced by a single agent may be deficient in aged animals or in animals endowed with short life spans. Such information would be crucial to a complete understanding of those processes leading to increased longevity. This concept could be applied to the work of Hart and Setlow[56] and Sacher and Hart[57] as well. Their studies of repair of U.V.-induced damage as a function of maximum life span capacity did not examine removal of specific lesions. Ultraviolet light-induced lesions include monomeric saturation of pyrimidine rings[143-145] and DNA-protein crosslinks[146] as well as pyrimidine dimers.[147] Differences in repair of any one of these might have produced the results they observed.

That DNA repair is a multifaceted process is emphasized by the discovery of the base excision and postreplication repair pathways. Either or both of these may be critically important to an understanding of aging.

Excision repair begins with endonucleolytic incision of DNA adjacent to a damaged region. In nucleotide excision repair, the incision is accomplished in one step. Base excision repair requires two steps for incision; first, cleavage of the base from its deoxyribose and second, incision of the DNA at or adjacent to the resulting apurinic site.[148]

Friedberg et al. have shown that mammalian cells contain uracil glycosidase activity. Such an activity could initiate the excision repair process by cleaving the glycosidic bond between a uracil in DNA and its corresponding deoxyribose.[148] This process would leave an apyrimidinic/apurinic site in the DNA. Endonucleases specifically recognizing apurinic sites have been isolated from rabbit and calf thymus,[149] rat liver,[150] and calf liver.[151] The repair process would continue by exonuclease removal of damaged residues, resynthesis of lost DNA by DNA polymerase, and ligation of the new and old DNA regions. These remaining processes are presumably indentical for nucleotide and base excision repair.

Note, as mentioned, that N7-ethylguanine, a product of ENU-induced DNA damage, and the BPADE-induced N7-guanine adduct are lost from the DNA primarily by depurination.[152,94] Repair of the resulting apurinic sites is expected to play a significant role in the repair processes monitored after treatment in this study.

Repair of apurinic sites in DNA may be of prime importance for determining the cellular error level and, therefore, the rate of natural aging of an organism. Lindahl[153] has estimated, from the rate constants for depurination and depyrimidation in native DNA in aqueous solution, that the average mammalian cell at 37°C is expected to lose 10,000 purines and 500 pyrimidines over a 20 hour period. These numbers are small compared to the total numbers of purines and pyrimidines in the DNA of a cell. However, they indicate that the DNA repair systems are called upon every day in every cell to perform thousands of repair operations. This occurs in addition to repair of damage from environmental sources. Also, in order to prevent errors from accumulating in the genome, the repair systems would need to have virtually 100% efficiency. Consider, for example, that 99% efficiency is routinely achieved. This encompasses both recognition of damage and fidelity of resynthesis. At 10,000 depurinations per day, 99% efficiency would leave an average of 100 errors in the genome of every cell in the entire organism. Over a year this would become 37,000 errors in the genome of every cell. Thus errors could easily accumulate in DNA, starting from birth, simply due to the aqueous and thermal environment of the DNA and to the lack of absolute perfection of the DNA repair process. A study of the efficiency of repair of apurinic sites as a function of the capacity for increased longevity may therefore be very appropriate.

Postreplication repair is usually defined operationally as the chasing of newly synthesized DNA into high molecular weight

DNA, as measured by alkaline sucrose gradient sedimentation, in spite of the presence of damaged bases in parental, template DNA during this process.[154] This is similar to the process measured in the experiments reported here. The major difference is in the DNA single-strand length being measured. In alkaline sucrose gradients, only DNA pieces below an upper limit of $5x10^8$ daltons are detectable.[154] Any changes in length above this limit are not observed. With the alkaline elution technique, the upper size limit is extended to $1.5x10^9$ daltons; increases in length can be followed up to this limit.[33] It has been extimated that rejoining of DNA to this length represents the joining of at least three replicon regions in the DNA.[84] Therefore, the elongation measurable in these experiments may not be entirely analogous to that usually referred to as postreplication repair.

Recent evidence suggests that a higher level of postreplication repair activity can be induced in mammalian cells by low levels of DNA damage.[155-157] In bacteria the inducible component of postreplication repair has been found to be error prone,[129] and, more recently, an inducible, error prone repair process has been reported in mammalian cells.[130] Specifically, it may be that damage not removed by the prereplication, excision repair processes is left to be dealt with by the postreplication repair mechanism. This may be processed without error by either a recombination mechanism or by hybridizing of daughter strands on homologous duplexes as discussed in Results. However, if the original level of damage is such that neither the prereplication nor the error-free, postreplication repair systems are able to deal with it effectively, the postreplication repair system may be forced to carry out an error producing process in order to replicate the DNA.[81] This latter process would correspond to the insertion of random bases opposite damaged regions of parental DNA as discussed in Results. The studies reported here may suggest increased capacity for postreplication repair in aged rats since there was greater daughter strand elongation in old donor cells in the presence of damaged, parental DNA. It is possible that cells from aged rats rely more heavily on postreplication repair to deal with damaged DNA and, therefore, are more likely to call on the error producing process than are cells from young rats. Note that these results seem to contradict the findings of Lehmann et al. of no alteration of postreplication repair with age in humans.[81] The techniques used here are expected to be much more sensitive than those used by Lehmann et al., and, as mentioned, the phenomena observed may not be precisely analogous.

That DNA repair is a multifaceted process is emphasized further by the finding that extracts of excision-deficient Xeroderma Pigmentosum fibroblasts are able to excise pyrimidine dimers from purified DNA but not from DNA in chromatin.[158] Apparently some component required for accessibility of repair enzymes to the DNA is missing or defective in these cells. It is possible that factors such as this or those responsible for subtle functions yet to be discovered could be involved in the aging process.

Another problem that deserves more study is that of mitochondrial DNA repair. Very little is known about this subject. In _Tetrahymena_ there are two DNA polymerase activities.[159-160] One is induced by U.V. irradiation, thymine starvation and treatment with ethidium bromide. The other appears to be maintained at constant levels, and, under normal conditions, constitutes the major DNA polymerase activity. The minor, inducible activity is associated with the mitochondrial fraction. Since it is induced by agents that cause DNA damage, it is tempting to speculate that this enzyme is involved in repair of mitochondrial DNA. Also, the induction is inhibited by cycloheximide and actinomycin D but not by chloramphenicol. Therefore, the inducible, mitochondrial DNA polymerase is synthesized by the nuclear-cytoplasmic system.

Repair of mitochondrial DNA is, apparently, difficult to demonstrate. Clayton et al.[161] searched for removal of pyrimidine dimer specific T4 endonuclease sites from human cells and found none. In their experiments, mitochondrial DNA was isolated, incubated with T4 endonuclease and then analyzed for nicking by the endonuclease using ethidium bromide-CsCl equilibrium centrifugation. Mitochondrial DNA was still nicked by the endonuclease 48 hours after U.V. irradiation of the cells. These results can be criticized, however, since the assay used could not distinguish between one and 50, for example, dimers in the closed, circular DNA. The actual number of dimers may have decreased from 50 to one during post-irradiation incubation, but the assay would have only shown that the DNA was still being nicked. On the other hand, repair of gamma-ray-induced single-strand breaks has been clearly demonstrated in Tetrahymena pyriformis.[162] Some evidence for repair of U.V.-induced damage in yeast mitochondria has also been obtained. After observing an increase in survival of normal colonies upon dark holding after high U.V. doses and a decrease in petite colonies upon dark holding after low U.V. doses, Hixon et al.[163] concluded that mitochondrial DNA was repaired in this system.

Mitochondrial DNA repair could be a key factor in mammalian aging. A buildup of errors in this system would lead to cellular senescence irregardless of the efficiency of repair in the nucleus.

To assure the relevance of a study of DNA repair to the natural aging process, it is important to understand how the damage created experimentally is related to that which occurs in vivo. Natural production of apurinic sites was discussed above. Unrepaired apurinic sites can lead to single-strand chain breakage with a $t_{1/2}$ of about 100 hours under physiological conditions.[164] They can also lead, albeit with low frequency, to the formation of cross links in DNA. This results since the ribose at an apurinic/apyrimidinic site is in equilibrium between the aldehyde and furanose form. It is the reactive aldehyde which can lead to cross link formation.[164]

Other pathways for damage production under physiological conditions include deamination of cytosine[165] and adenine[166] to yield uracil and hypoxanthine, respectively, and opening of the imidazole ring of adenine.[167] The isolation of a mammalian endonuclease specific for uracil in DNA points out the significance of cytosine deamination for cell viability.[148]

Another principal source of damage to DNA, and other cellular components as well, is from the action of free radicals and peroxides produced by normal metabolic processes[168] and the metabolism of certain chemicals.[169] These species, especially hydroxyl free radicals which are produced upon reaction of superoxide anion with peroxides,[170] would be expected to produce the same types of damage produced by ionizing radiation which yields hydroxyl radicals upon interaction with water.[171] These include DNA-protein crosslinks,[138] and DNA base damage and single-strand breaks.[172] Double-strand breaks could be effectively accomplished by production of single-strand breaks in close proximity on opposite strands.

Besides metabolic processes, another important source of free radical damage is background radiation from radionuclides contained within the cell. Neutrons produced by interaction of cosmic radiation with our upper atmosphere react with ^{14}N to produce ^{3}H and ^{14}C on a steady, continuous basis.[173] These radionuclides mix with the biosphere to maintain a steady-state level of each in all systems that are in equilibrium with the environment; including man. The level of ^{14}C, for example, is maintained at about 15 DPM per gram of carbon in all equilibrating systems.[174] When an animal dies, it no longer equilibrates with the environment. The ^{14}C that was in the tissues at death decays with time, and, therefore, the decrease in DPM per gram of carbon is one way of determining the time of death of an organism.

Not only do ^{3}H and ^{14}C produce ionizing radiation in the form of beta particles, but they are expected to produce damage due to the nature and location of their decay products. For example, single-strand breaks arise from decay of (2-^{3}H) adenine contained in DNA due to emission of beta particles while DNA-DNA crosslinks are caused by local effects of the decay other than beta emission.[175] Decay of (5-^{3}H) cytosine in DNA leads to formation of uracil.[176] This is thought to occur by decay of ^{3}H to yield (5-^{3}He) cytosine which would carry a positive charge. Subsequent elimination of

^{3}He and attack of the positively charged pyrimidine ring by hydroxyl anion would yield (5-OH) cytosine which would spontaneously deaminate to yield uracil.[176] Local effects due to transformation of ^{14}C to ^{14}N might be expected to lead to ring fragmentation and/or abnormal base pairing relationships. Consider, for example, (6-^{14}C) guanine. When this decays the resulting (6-^{14}N) guanine would probably be found only as the enol tautomer, thereby disrupting normal base pairing.

Another major source of natural, intracellular radiation is ^{40}K. It is from ^{40}K that we receive our highest dose of background radiation.[177] The combined dose rate of beta and gamma radiation from ^{40}K is about 30 times higher than that from ^{14}C.

Free radical damage to other macromolecules, such as membrane lipids, may also lead to DNA damage. Lipid autoxidation has been shown to damage DNA in artificial systems.[178] In the cell this could occur by the juxtaposition of DNA and lipid as is found at the nuclear envelope. Free radical damage to lipids initiates a chain reaction whereby the free radical is transferred from one unsaturated lipid side chain to another in continuous layers of lipid as found in cell membranes. It has been estimated that chain reactions such as this have a range up to 0.001 cm.[179] If a strand of DNA is adjacent to the lipid bilayer of the nuclear envelope when a free radical is "passing by", the free radical could attact the DNA rather than another lipid side chain.

Spontaneous depurination, metabolic production of free radicals, and internal sources of ionizing radiation could thus constitute a significant, natural source of damage to DNA and other cellular components. It may have been these agents and the damage they produce that had to be dealt with in order for our ancestral line to effect an increase in life span.[1]

As mentioned, ionizing radiation produces, via hydroxyl radicals, several types of DNA damage. Monomeric saturation of pyrimidine rings would lead to minimal helix distortion while DNA-DNA and DNA-protein crosslinks could create maximal helix distortion.[172] Free radical-induced damage may thus be compared to ENU and N-AAAF-induced damage, respectively. Similarly, ultraviolet light, as used in the Hart and Setlow[56] and Sacher and Hart studies,[57] produces monomeric saturation of pyrimidine rings[143-145] and DNA-protein crosslinks[146,139] in addition to pyrimidine dimers,[147] as mentioned earlier. The former would produce a low degree and the latter two a high degree of helix distortion. Therefore, it can be appreciated that, in fact, experimentally induced damages are seemingly appropriate models of naturally occurring DNA damage.

Evidence of decreased DNA repair in aged animals leads naturally to the following question. Does DNA repair capacity decline at a constant rate throughout life from birth or is there a point in life where the decline is initiated or hastened? Discovery of some event in development which hastens the decline of processes for repair and/or protection of cellular components could lead to our intervention with the mechanism involved so as to decrease the rate of aging thereafter.

As Cutler[85] has pointed out, there are probably two levels of processes determining the rate of aging. At the cellular level are those processes serving to protect the cell from natural, internal sources of damage and those for repairing damage after it occurs. At the systemic level are those factors which modulate the protective and/or repair processes within the cells.

Calow[180] has argued that accumulation of error containing macromolecules in aging organisms is likely to be due to declining activity of repair systems which would be expected to accumulate errors as well. However, he argues that the decline in repair activity is the by-product result of some process necessary during the development of the organism. Bullough[181] has discussed evidence suggesting that as cells mature and differentiate, they are commmitted to a pathway leading to senescence and death. This can be seen, for instance, in the differentiation and death of epidermal cells after division of the basal, stem cells. While this system is subject to rather rapid turnover, analogy can be drawn to other systems with long or infinite turnover times such as nervous tissue and skeletal muscle. It may be that the events leading to maturation, with its attendent slowing down of cell growth and turnover, somehow produce, as a side effect, an inhibition of repair and protection processes so as to insure the accumulation of error-containing components and, therefore, the senescence of the organism.

Inhibition of DNA repair upon differentiation of nerve and muscle cells was discussed earlier. Additional evidence points to likely sources of further inhibition of repair and/or protective processes during development. Denckla,[182] for instance, has demonstrated the existence of a pituitary factor that lowers the minimal oxygen consumption (MOC) of rats. This factor is apparently produced beginning at puberty and blocks the stimulation of the

MOC by thyroxin. The MOC in rats increases until puperty and declines thereafter. In humans, even though the serum protein bound iodine (PBI) remains constant with increasing age, the basal metabolic rate (BMR) decreases with age in adults.[183] This situation is exactly what would be expected in humans if, as has been suggested,[184] the pituitary-thyroid axis serves only as a servomechanism to maintain the level of circulating thyroid hormone at a constant level. If a factor is produced by the human pituitary similar to that in rats, the PBI would be expected to remain constant, but the effect of thyroxin on the tissues, reflected in the BMR, would be expected to decrease at puberty. To offer an explanation for the continued decrease in BMR with advancing age, we must look at the action of thyroxin at the cellular level.

Although the cellular effects of thyroxine are not limited to mitochondria, Tata et al.[185] have shown that thyroxin at physiological doses, stimulates both mitochondrial respiration and oxidative phosphorylation in rats. Clearly then, thyroxine would have an affect on the availability of useable energy in a cell. Let us assume for the sake of argument that a certain level of stimulation by thyroxin is necessary to maintain the proper energy level in a cell. We know that energy is required by all of the DNA repair processes either directly in the process of repair or indirectly in the synthesis of the repair systems. If the energy supply of a cell is diminished by something that blocks the required stimulation by thyroxin, we can see that the error level in that cell might increase. In fact, it is not hard to imagine that, in this situation, the error level might snowball. The normal processes that maintain the error level at an acceptable level would be hampered in their function and so themselves become more error laden. This would fit in very nicely with Holliday's[6] concept discussed in the Introduction. The decrease in energy availability would cause the probability of a given cell becoming committed to an irreversible sequence of events leading to death to increase to an unacceptable level. This, in turn, would guarantee the senescence and ultimate death of the organism.

By removing the pituitary from rats and giving replacement thyroid therapy, Denckla was able to restore the MOC of adult rats to levels found in young, prepubertal rats.[182] Further, Bilder and Denckla were able to reverse age-associated declines in graft rejection and reticuloendothelial function of rats similarly treated.[186]

Recent evidence also indicates that a substance from the pituitary inhibits peripheral responsiveness to growth hormone.[187] When the pituitary is removed from rats, the peripheral response to growth hormone returns. Both the thyroid hormone blocking factor and the growth hormone blocking factor have long biological half lives of about six months.[187] It is tempting to speculate that they may be the same or similar factors.

Study of the relation between DNA repair and aging will not be complete, in my opinion, until the possible role of hormonal events occurring at puberty in modulating DNA repair have been assessed. Also, as discussed, there are many facets of DNA repair which remain to be studied. Certain of these may be more important in determining the rate of aging than others, and certain aspects may be subject to a greater degree of modulation by hormonal events. Resistance of cells to DNA damaging insults is only one part of the overall scheme for protection and repair of cellular damage. All of these mechanisms need to be studied in relation to maximum life span capacity, as well as possible modulation of these functions during development and senescence.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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